

STARTER DISTILLATE #86  
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**VOLUME 2**

# **GRAS MONOGRAPH SERIES**

## **STARTER DISTILLATE**

**(COPIES OF ARTICLES CITED IN  
MONOGRAPH SUMMARY)**

**prepared for**  
**THE FOOD AND DRUG ADMINISTRATION**  
**DEPARTMENT OF HEALTH, EDUCATION**  
**AND WELFARE**

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**prepared by**  
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Tuesday, May 9, 1961

## FEDERAL REGISTER

3991

## Title 21—FOOD AND DRUGS

## Chapter I—Food and Drug Administration, Department of Health, Education, and Welfare

## SUBCHAPTER B—FOOD AND FOOD PRODUCTS

## PART 121—FOOD ADDITIVES

## Subpart B—Exemption of Certain Food Additives From the Requirement of Tolerances

## SUBSTANCES THAT ARE GENERALLY RECOGNIZED AS SAFE

Pursuant to the authority vested in the Secretary of Health, Education, and Welfare by the Federal Food, Drug, and Cosmetic Act (secs. 409, 701, 72 Stat. 1785, 52 Stat. 1055 as amended; 21 U.S.C. 348, 371), and delegated to the Commissioner of Food and Drugs by the Secretary (25 F.R. 8625), and after having considered all data accumulated with reference to the proposed order published in the FEDERAL REGISTER of August 12, 1960 (25 F.R. 7698), containing a list of synthetic flavoring substances regarded as generally recognized as safe within the meaning of section 409 of the act, the Commissioner has concluded that the substances in that list are safe for their intended use. Therefore, it is ordered, That the food additive regulations (21 CFR 121.101) be amended by adding to § 121.101 the following new paragraph (g):

§ 121.101 Substances that are generally recognized as safe.

(g) Synthetic flavoring substances that are generally recognized as safe for their intended use, within the meaning of section 409 of the act, are as follows:

Acetaldehyde (ethanal).  
Acetoin (acetyl methylcarbinol).  
Acornic acid (equisetinic acid, citridic acid, achillic acid).  
Anethole (parapropenyl anisole).  
Benzaldehyde (benzoic aldehyde).  
Brominated vegetable oils.  
N-Butyric acid (butanoic acid).  
d- or l-Carvone (carvol).  
Cinnamaldehyde (cinnamic aldehyde).  
Citral (2,6-dimethyloctadien-2,6-di-al-8, geraniol, neral).  
Decanal (N-decylaldehyde, capraldehyde, capric aldehyde, caprinaldehyde, aldehyde C-10).  
Diacetyl (2,3-butanedione).  
Ethyl acetate.  
Ethyl butyrate (so-called rum ether).  
3-Methyl-3-phenyl glycidic acid ethyl ester (ethyl-methyl-phenyl-glycidate, so-called strawberry aldehyde, C-18 aldehyde).  
Ethyl vanillin.  
Eugenol.  
Geraniol (3,7-dimethyl-2,6 and 3,6-octadien-1-ol).  
Geranyl acetate (geraniol acetate).  
Glycerol (glyceryl) tributyrate (tributyrin, butyrin).  
Limonene (d-, l-, and dl-).  
Linalool (linalol, 3,7-dimethyl-1,6-octadien-3-ol).  
Linalyl acetate (bergamot).  
l-Malic acid.  
Methyl anthranilate (methyl-2-aminobenzoate).  
Piperonal (3,4-methylenedioxy-benzaldehyde, heliotropin).  
Vanillin.

**Effective date.** This order shall become effective 30 days from the date of its publication in the FEDERAL REGISTER.

(Secs. 409, 701, 72 Stat. 1785, 52 Stat. 1055, as amended; 21 U.S.C. 348, 371)

Dated: May 2, 1961.

[SEAL] GEO. P. LARRICK,  
Commissioner of Food and Drugs.

[F.R. Doc. 61-4228; Filed, May 8, 1961; 8:48 a.m.]

## PART 121—FOOD ADDITIVES

## Subpart D—Food Additives Permitted in Food for Human Consumption

## HYDROXYLATED LECITHIN

The Commissioner of Food and Drugs, having evaluated the data submitted in a petition by Food Technology, Inc., 5903 Northwest Highway, Chicago 31, Illinois, and other relevant material, has concluded that the following amendment should issue in conformance with section 409 of the Federal Food, Drug, and Cosmetic Act, with respect to the food additive hydroxylated lecithin to provide for an alternative method of manufacture. Therefore, pursuant to the provisions of the act (sec. 409(c)(1), 72 Stat. 1786; 21 U.S.C. 348(c)(1)) and under the authority delegated to the Commissioner by the Secretary of Health, Education, and Welfare (25 F.R. 8625), § 121.1027(a) of the food additive regulations (21 CFR 121.1027; 25 F.R. 13217) is amended to read as follows:

§ 121.1027 Hydroxylated lecithin.

(a) The additive is obtained by the treatment of lecithin in one of the following ways, under controlled conditions whereby the separated fatty acid fraction of the resultant product has an acetyl value of 30 to 38:

(1) With hydrogen peroxide, benzoyl peroxide, lactic acid, and sodium hydroxide.

(2) With hydrogen peroxide, acetic acid, and sodium hydroxide.

Any person who will be adversely affected by the foregoing order may at any time prior to the thirtieth day from the date of its publication in the FEDERAL REGISTER file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 5440, 330 Independence Avenue SW., Washington 25, D.C., written objections thereto. Objections shall show wherein the person filing will be adversely affected by the order and specify with particularity the provisions of the order deemed objectionable and the grounds for the objections. If a hearing

is requested, the objections must state the issues for the hearing. A hearing will be granted if the objections are supported by grounds legally sufficient to justify the relief sought. Objections may be accompanied by a memorandum or brief in support thereof. All documents shall be filed in quintuplicate.

**Effective date.** This order shall be effective on the date of its publication in the FEDERAL REGISTER.

(Sec. 409(c)(1), 72 Stat. 1786; 21 U.S.C. 348(c)(1))

Dated: May 2, 1961.

[SEAL] GEO. P. LARRICK,  
Commissioner of Food and Drugs.

[F.R. Doc. 61-4229; Filed, May 8, 1961; 8:48 a.m.]

## SUBCHAPTER C—DRUGS

## PART 141a—PENICILLIN AND PENICILLIN-CONTAINING DRUGS; TESTS AND METHODS OF ASSAY

## PART 146a—CERTIFICATION OF PENICILLIN AND PENICILLIN-CONTAINING DRUGS

## Miscellaneous Amendments

Under the authority vested in the Secretary of Health, Education, and Welfare by the Federal Food, Drug, and Cosmetic Act (sec. 507, 59 Stat. 463, as amended; 21 U.S.C. 357) and delegated to the Commissioner of Food and Drugs by the Secretary (25 F.R. 8625), the regulations for tests and methods of assay and certification of penicillin and penicillin-containing drugs (21 CFR Parts 141a, 146a) are amended as follows:

1. Section 141a.1 *Sodium penicillin* \* \* \* is amended in the following respects:

a. Paragraph (c) is amended by changing the third sentence to read: "Keep this solution under refrigeration and use for 2 days only."

b. Paragraph (e) is amended by changing the last two sentences to read as follows: "Determine by appropriate tests the quantity of this resulting dilution to be added to each 100 milliliters of agar, which has been melted and cooled to 48° C., for the secondary layer that will give sharp, clear zones of inhibition. The suspension may be used for 1 week."

2. Section 141a.5 *Sodium penicillin* \* \* \* is amended in the following respects:

a. The first equation at the end of paragraph (d) (1) is changed to read:

$$\text{Units of penicillin G per milligram} = \frac{\text{Difference in titers} \times \text{potency of FDA penicillin G working standard in units per milligram}}{\text{Sample weight (milligrams) in 2.0 milliliters} \times F}$$

b. Paragraph (g) is amended by changing the last three sentences and the equation to read as follows: "Determine the absorbance of the blank compared with 0.1 N NH<sub>4</sub>OH at 220 mμ

and 224 mμ. Determine the absorbance of the sample at 220 mμ and 224 mμ compared with the blank. Calculate the quantity of benzoic acid in the solution from the equation:

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to the proposal, and other available information have been considered, and it is concluded that it will promote honesty and fair dealing in the interest of consumers to adopt the amendments as set forth below.

Therefore, pursuant to the authority vested in the Secretary of Health, Education, and Welfare by the Federal Food, Drug, and Cosmetic Act (secs. 401, 701, 52 Stat. 1046, 1055, as amended 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371) and delegated by him to the Commissioner of Food and Drugs (21 CFR 2.120): It is ordered, That § 19.530 be amended by revising paragraph (b) (4), by adding a new subparagraph (7) to paragraph (b), by redesignating paragraph (d) (2) as (d) (3), with changes, and by adding to paragraph (d) a new subparagraph (2). The affected portions read as follows:

§ 19.530 Creamed cottage cheese; identity; label statement of optional ingredients.

(b) . . . .

(4) A preparation of pasteurized skim milk or cottage cheese whey with added citric acid or sodium citrate, which preparation has been cultured with harmless flavor- and aroma-producing bacteria.

(7) Singly or in combination: Diacetyl, starter distillate, or other safe and suitable flavoring substances which contribute to the characteristic flavor and aroma associated with the food.

(d) . . . .

(2) When any ingredient named under paragraph (b) (7) is used, the label shall bear the statement "artificially flavored" or "artificial flavor added" or "with added artificial flavoring".

(3) Wherever the name "creamed cottage cheese" appears on the label so conspicuously as to be easily seen under customary conditions of purchase, the label declarations prescribed in subparagraphs (1) and (2) of this paragraph, showing the optional ingredients present, shall immediately and conspicuously precede or follow such name without intervening written, printed, or graphic matter.

Any person who will be adversely affected by the foregoing order may at any time within 30 days following the date of its publication in the FEDERAL REGISTER file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 5440, 330 Independence Avenue SW., Washington, D.C. 20201, written objections thereto. Objections shall show wherein the person filing will be adversely affected by the order and specify with particularity the provisions of the order deemed objectionable and the grounds for the objections. If a hearing is requested, the objections must state the issues for the hearing, and such objections must be supported by grounds legally sufficient to justify the relief sought. Objections may be accompanied by a memorandum or brief in support

thereof. All documents shall be filed in six copies.

*Effective date.* This order shall become effective 60 days from the date of its publication in the FEDERAL REGISTER, except as to any provisions that may be stayed by the filing of proper objections. Notice of the filing of objections or lack thereof will be announced by publication in the FEDERAL REGISTER.

(Secs. 401, 701, 52 Stat. 1046, 1055, as amended 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371)

Dated: May 9, 1967.

JAMES L. GODDARD,  
Commissioner of Food and Drugs.

[F.R. Doc. 67-5443; Filed, May 15, 1967;  
8:50 a.m.]

Title 21—FOOD AND DRUGS

Chapter I—Food and Drug Administration, Department of Health, Education, and Welfare

SUBCHAPTER B—FOOD AND FOOD PRODUCTS

PART 19—CHEESES, PROCESSED CHEESES, CHEESE FOODS, CHEESE SPREADS, AND RELATED FOODS

Creamed Cottage Cheese; Order Amending Identity Standard to List Diacetyl and Other Flavors; Cottage Cheese Whey, and Sodium Citrate as Optional Ingredients

In the matter of amending the standard of identity for creamed cottage cheese (21 CFR 19.530) by listing diacetyl, starter distillate, and other safe and suitable flavoring substances that enhance the characteristic flavor and aroma of the food as optional ingredients of the creaming mixture; and by listing cottage cheese whey and sodium citrate to provide another citrated medium in which to culture flavor- and aroma-producing bacteria for addition to the creaming mixture:

A notice of proposed rulemaking in the above-identified matter was published in the FEDERAL REGISTER of February 8, 1967 (32 F.R. 2646), based on a petition submitted by the Milk Industry Foundation, 1012 14th Street NW., Washington, D.C. 20005.

The information furnished by the petitioner, the comments filed in response



9 C R, 1973

Chapter III—Animal, Plant Health Inspection Serv. (Meat, Poultry)

§ 318.7

Class of substance	Substance	Purpose	Products	Amount
	Polyglycerol esters of fatty acids (polyglycerol esters of fatty acids are restricted to those up to and including the decaglycerol esters and otherwise meeting the requirements of § 121.1120 (a) of the Food Additive Regulations).	.....do.....	Rendered animal fat or a combination of such fat with vegetable fat when use is not precluded by standards of identity or composition.	Sufficient for purpose.
	Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate).	.....do.....	Shortening for use in nonstandardized baked goods, baking mixes, icings, fillings, and toppings and in the frying of foods.	1 percent when used alone. If used with polysorbate 60 the combined total shall not exceed 1 percent.
	Propylene glycol mono and diesters of fats and fatty acids.	.....do.....	Rendered animal fat or a combination of such fat with vegetable fat.	Sufficient for purpose.
	Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate).	.....do.....	Shortening for use in nonstandardized baked goods, baking mixes, icings, fillings, and toppings and in the frying of foods.	1 percent when used alone. If used with polysorbate 80 the combined total shall not exceed 1 percent.
	Steryl-2-lactylic acid.	.....do.....	Shortening to be used for cake icings and fillings.	3.0 percent.
	Steryl monoglyceridyl citrate.	.....do.....	Shortening.	Sufficient for purpose.
Flavoring agents; protectors and developers.	Program approved artificial smoke flavoring. <sup>1</sup>	To flavor product...	Various <sup>2</sup> .....	Do.
	Program approved smoke flavoring. <sup>1</sup>	.....do.....	.....do.....	Do.
	Autolyzed yeast extract.	.....do.....	.....do.....	Do.
	Harmless bacteria starters of the acidophilus type, lactic acid starter or culture of <i>Pedococcus cerevisiae</i> .	To develop flavor...	Dry sausage, pork roll, thuringer, lebanon bologna, cervelat, and salami.	0.5 percent.
	Benzole acid, sodium benzoate.	To retard flavor reversion.	Oleomargarine.....	0.1 percent.
	Citric acid.	To protect flavor	.....do.....	Sufficient for purpose.
	.....do.....	Flavoring.....	Chili con carne.	Sufficient for purpose.
	Corn syrup solids, corn syrup, glucose syrup.	To flavor.....	Sausage, hamburger, meat loaf, luncheon meat, chopped or pressed ham.	2.0 percent individually or collectively, calculated on a dry basis.
	Dextrose.....	To flavor product...	Sausage, ham and cured products.	Sufficient for purpose.
	Diacetyl.....	.....do.....	Oleomargarine.....	Do.
	Disodium guanylate.....	.....do.....	Various <sup>2</sup> .....	Do.
	Disodium inosinate.....	.....do.....	Various <sup>2</sup> .....	Do.
	Hydrolyzed plant protein.	.....do.....	Various <sup>2</sup> .....	Do.
	Isopropyl citrate.....	To protect flavor	Oleomargarine.....	0.02 percent.
	Malt syrup.....	To flavor product...	Cured products.....	2.5 percent.
	Milk protein hydrolysate.	.....do.....	Various <sup>2</sup> .....	Sufficient for purpose.
	Monosodium glutamate.	.....do.....	.....do.....	Do.
	Sodium sulfacetate derivative of mono and diglycerides.	.....do.....	.....do.....	0.5 percent.

§ 318.7

## Title 9—Animals and Animal Products

Class of substance	Substance	Purpose	Products	Amount
	Sodium tripolyphosphate.	To help protect flavor.	"Fresh Beef," "Beef for Further Cooking," "Cooked Beef," and similar products which are frozen after processing.	Do.
	Mixtures of sodium triphosphate and sodium hexametaphosphate.	.....do.....	.....do.....	Do.
	Sorbitol.....	To flavor, to facilitate the removal of casings from product and to reduce caramelization and charring.	Cooked sausage labeled frankfurter, frank, further, wiener, knock-wurst.	Not more than 2 percent the weight of the formula excluding the weight of water or not permitted in combination with corn syrup and/or corn syrup solids.
	Starter distillate.....	To help protect flavor.	Oleomargarine.....	Sufficient for purpose.
	Stearyl citrate.....	To protect flavor.....	.....do.....	0.15 percent.
	Sugars (sucrose and dextrose).	To flavor product.....	Various.....	Sufficient for purpose.
Cases.....	Carbon dioxide solid (dry ice).	To cool product.....	Chopping of meat, packaging of product.	Do.
Hog scald agents; must be removed by subsequent cleaning operations.	Nitrogen.....	To exclude oxygen.....	Sealed container.....	Do.
	Caustic soda.....	To remove hair.....	Hog carcasses.....	Do.
	Dioctyl sodium sulfosuccinate.	.....do.....	.....do.....	Do.
	Lime.....	.....do.....	.....do.....	Do.
	Methyl polysilicone.....	.....do.....	.....do.....	Do.
	Sodium carbonate.....	.....do.....	.....do.....	Do.
	Sodium dodecylbenzene sulfonate.	.....do.....	.....do.....	Do.
	Sodium hexametaphosphate.	.....do.....	.....do.....	Do.
	Sodium lauryl sulfate.	.....do.....	.....do.....	Do.
	Sodium metasilicate.	.....do.....	.....do.....	Do.
	Sodium n-alkylbenzene sulfonate (alkyl group predominantly C <sub>12</sub> and C <sub>14</sub> and not less than 95 percent C <sub>10</sub> to C <sub>16</sub> ).	.....do.....	.....do.....	Do.
	Sodium sulfate.	.....do.....	.....do.....	Do.
	Sodium tripolyphosphate.	.....do.....	.....do.....	Do.
	Sucrose.....	.....do.....	.....do.....	Do.
	Trisodium phosphate.	.....do.....	.....do.....	Do.
Miscellaneous.....	Potassium sorbate.....	To retard mold growth.	Dry sausage.....	2.5 percent in water solution may be applied to casings after stuffing; casings may be dissolved prior to stuffing.
		To preserve product and to retard mold growth.	Oleomargarine or margarine.	0.1 percent by weight of the finished oleomargarine or margarine.
	Calcium disodium, EDTA (calcium disodium ethylenediaminetetraacetate).	To preserve product and to protect flavor.	.....do.....	75 parts per million by weight of the finished oleomargarine or margarine.
	Propyl paraben (propyl p-hydroxybenzoate).	To retard mold growth.	Dry sausage.....	3.5 percent in water solution may be applied to casings after stuffing or casings may be dissolved prior to stuffing.
	Sodium bicarbonate....	To neutralize excess acidity, cleaning vegetables.	Rendered fats, soups, curing pickle.	Sufficient for purpose.

See footnotes at end of table.

Table I

Groups, of 16 male Sprague-Dawley rats each, were fed a riboflavin-low semi-synthetic diet<sup>1</sup>, containing 0.06% 3'-Me-DAB, for periods varying from 1 to 12 weeks. After the respective periods of dye feeding the animals were continued to be fed the same diet without dye, and were sacrificed after a total of 7 months<sup>2</sup>.

Weeks of feeding	No. of tumor bearers over total survivors	Percentage of tumor incidence
1	0/14	0 %
2	0/15	0 %
3	1/13	7.5 %
4	4/16	25 %
5	8/16	50 %
6	11/17	65 %
8	16/16	100 %
10	16/16	100 %

Experientia (Basel) 15: 316-317, (1959)  
 Studies on the Swelling of Rat-Liver  
 Mitochondria in Relation to Tumor  
 Incidence During Feeding of Aminoazo Dyes

It was reported previously by one of us<sup>1</sup> that the swelling ability of rat liver microsomes decreases during feeding of 3'-methyl-*p*-dimethylaminoazobenzene (3'-Me-DAB), reaching a minimum level at 4 weeks. Even though the feeding of the dye is continued, this swelling ability eventually recovers and reaches a normal level at about 20 weeks. The microsomes from hepatoma, induced with 3'-Me-DAB, swell to an extent comparable to those of the liver after 4 weeks of feeding of this same dye. The non-carcinogenic isomer, 2-Me-DAB, however, does not produce these effects.

This report<sup>2</sup> describes a correlation that has now been established between microsomal swelling and the appearance of gross tumors. Table I shows that if the feeding of 3'-Me-DAB to rats is continued beyond 4 weeks under our experimental conditions, the animals rapidly reach a point of no return, since the percentage of tumor incidence in the groups shows a sudden steep rise at about 4 weeks.

The fine structural alterations in the cell, that can be detected by the study of swelling, during chemical carcinogenesis are, however, not restricted to the endoplasmic reticulum, origin of the 'microsome' fraction. Of more particular interest in this respect are the findings of EMMELOT and BOS, that the thyroxine-induced swelling of rat liver mitochondria decreases after feeding DAB for 5 months<sup>3</sup> or by incubating these particulates *in vitro* with carcinogens<sup>4</sup>.

Table II shows alterations of the mitochondrial swelling, essentially similar to those of microsomal swelling during feeding 3'-Me-DAB<sup>1</sup>. There is a minimum swelling with the liver at about 4 weeks, and low values have been observed with the hepatoma. As with the microsomes<sup>1</sup>, no appreciable change in mitochondrial swelling was observed during feeding 0.06% of the non-carcinogenic dye, 2-Me-DAB, for 6 weeks.

<sup>1</sup> J. C. AREAS and M. AREAS, *Biochim. biophys. Acta* 28, 9 (1958); *Naturwissenschaften* 44, 331 (1957).

<sup>2</sup> These investigations are supported by the U. S. Public Health Service Grant C-1351.

<sup>3</sup> P. EMMELOT and C. J. BOS, *J. exp. Cell Res.* 12, 191 (1957).

<sup>4</sup> P. EMMELOT and C. J. BOS, *Biochim. biophys. Acta* 24, 412 (1957).

The differences between the macromolecular organization of liver mitochondria and of hepatoma mitochondria are evident not only from the extent of swelling, but also from its pH dependence (cf. <sup>1</sup>). Swelling was studied in 0.17 *M* sucrose, and in 0.30 *M* sucrose in presence of  $1 \times 10^{-5}$  *M*/l thyroxine or of  $5 \times 10^{-3}$  *M*/l  $\text{CaCl}_2$ . The pH range was from 5.0 to 9.6. All curves obtained with liver mitochondria show relatively sharp maxima around pH 7.4, while the curves obtained with the hepatoma were rather flat with no clear-cut maxima.

Table II

Animals and diet were identical to those described in Table I. The method of isolation of the mitochondria and the swelling test (40 min) were essentially those described by TAPLEY<sup>5</sup>. Percentage swelling was calculated as in previous work<sup>1</sup>.

'Percentage Swelling'

Weeks of dye feeding	0.17 <i>M</i> sucrose	0.30 <i>M</i> sucrose $1 \times 10^{-5}$ thyroxine	0.30 <i>M</i> sucrose $5 \times 10^{-3}$ $\text{CaCl}_2$	0.30 <i>M</i> sucrose $1 \times 10^{-3}$ $\text{HgCl}_2$
0	50.2	37.2	38.0	65.1
2	44.1	24.1	46.5	55.8
3	—	28.1	41.4	50.2
4	35.0	13.8	29.9	29.4
5	—	9.3	32.3	30.9
8	—	19.2	36.2	35.9
10	38.6	21.2	37.1	—
12	—	29.8	41.4	47.6
Tumor	18.7	12.1	19.7	21.3

The influence of various compounds on the swelling on normal mitochondria was studied with particular emphasis on the role of sulfhydryl groups. Since carbonyl compounds are known to interact with sulfhydryl groups of proteins, the effect of such agents was investigated. The fact, that at the same molar concentration of  $1 \times 10^{-2}$  only alloxan and diacetyl are inhibitors of swelling, while acetone, chloroacetone, acetylacetone, and acetonylacetone are inactive, seems to indicate that at least two vicinal carbonyl groups in the molecule are required for the inhibition of mitochondrial swelling by these com-

<sup>5</sup> The authors wish to thank Dr. V. M. AREAN and Dr. J. SIMON for the histopathological examinations.

<sup>6</sup> D. F. TAPLEY, *J. biol. Chem.* 222, 325 (1956).

pounds. The inhibition caused by diacetyl is abolished when the rats are fed 0.06% 3'-Me-DAB over 4 weeks.

Sulfhydryl compounds may have enhancing or inhibiting effect on mitochondrial swelling which suggests that it is not the mere presence of -SH groups or the reducing properties that are determining, but rather the general structural pattern of the whole molecule. In fact, at the same molar concentration of  $1 \times 10^{-3}$  reduced glutathione causes considerable enhancement of swelling (cf. <sup>7</sup>), sodium thiosulfate has no effect, while 2,3-dimercaptopropanol causes nearly total inhibition.  $\alpha$ -Lipoic acid gives some enhancement at  $1 \times 10^{-3}$  and at  $5 \times 10^{-3}$  M/l, while the unsubstituted *n*-octanoic acid causes total inhibition at  $5 \times 10^{-3}$  M/l.

Tentatively the following mechanism is suggested for the action of azo-dyes at the level of the mitochondria: The many structurally unrelated biological agents, which affect the rate of metabolism, may do so by acting on reversible structural changes in the dynamic mitochondrial membrane (e.g. <sup>8</sup>). The azo-carcinogens act at this level by inhibiting swelling, possibly through cross-linking of the elastic membrane (cf. <sup>9</sup>). Thus, the membrane may escape certain metabolic regulatory stimuli because of an acquired greater structural rigidity, that is a new macromolecular pattern which, once established, is transmitted to the subsequent generations of cells. The large increase of cystine content of the mitochondria, when passing from the liver to the hepatoma<sup>10</sup> is not inconsistent with this concept.

A full account of these and related investigations will be given elsewhere.

J. C. ARCOS, G.W. GRIFFITH, and R.W. CUNNINGHAM

*Cancer Research Laboratory, The J. Hillis Miller Health Center, University of Florida, Gainesville, April 9, 1959.*

#### Résumé

Dans des groupes de rats nourris à un régime contenant du 3'-méthyl-*p*-diméthylaminoazobenzène, on constate, qu'il y a un accroissement soudain, à 4 semaines, de l'incidence de tumeurs hépatiques. Cette observation établit une corrélation directe entre la cancérogenèse et le minimum des courbes de gonflement des microsomes et des mitochondries de foies de rats, nourris dans les mêmes conditions.

<sup>7</sup> A. L. LEHNINGER and M. SCHNEIDER, J. biophys. biochem. Cytol. 5, 109 (1959).

<sup>8</sup> P. SIEKEVITZ and M. WATSON, J. biophys. biochem. Cytol. 2, 639 (1956).

<sup>9</sup> M. ARCOS and J. C. ARCOS, *Atzneimittelforschung* 8, 643 (1958).

<sup>10</sup> B. S. SCHWEIGERT, B. T. GUTHNECK, J. M. PRICE, J. A. MILLER, and E. C. MILLER, Proc. Soc. exp. Biol. Med., N.Y. 72, 495 (1949).

*J. Biophysic. biochem. Cytol.* 7:49-60, (1960)  
 Fine Structural Alterations in Cell Particles During  
 Chemical Carcinogenesis

II. Further Evidence for Their Involvement in the Mechanism of Carcinogenesis. The Swelling of Rat Liver Mitochondria During Feeding of Amino Azo Dyes\*

By JOSEPH C. ARCOS, D.Sc., GEORGE W. GRIFFITH, and RICHARD W. CUNNINGHAM  
 (From the Cancer Research Laboratory, The J. Hillis Miller Health Center, University of Florida, Gainesville)

(Received for publication, July 15, 1959)

ABSTRACT

Swelling under carefully controlled conditions has been used to study alterations in the structure of rat liver mitochondria as a result of feeding azo dyes. The changes of the swelling properties of the mitochondria during feeding of the hepatocarcinogenic 3'-methyl-4-dimethylaminoazobenzene are essentially comparable to those observed previously with the microsomes, under the same dietary conditions. These alterations in mitochondrial swelling are not related to changes in the amount of these cell particulates per unit weight of tissue, during feeding of this azo dye. As with the microsomes, feeding of the isomeric but relatively non-carcinogenic 2-methyl-4-dimethylaminoazobenzene does not affect swelling. The structural differences between liver and hepatoma mitochondria show up not only in the rate and extent of swelling but also in the form of the curves of pH dependence.

The influence of ketones and sulphydryl compounds on the swelling of normal liver mitochondria were studied, with particular emphasis to the role of sulphydryl groups in membrane permeability.

The sudden steep rise in the tumor incidence in groups of rats fed 3'-methyl-4-dimethylaminoazobenzene for increasing intervals of time occurs at about 4 weeks. This time correlates with the point of the minimum swelling of microsomes and mitochondria isolated from the livers of rats fed this same dye. Thus, a correlation is established between the alterations of the swelling properties of these particulates and the carcinogenic process.

In previous studies (1) swelling was used to detect alterations in the fine structure of rat liver microsomes during feeding of 3'-methyl-4-dimethylaminoazobenzene. It was observed that the increase of the swelling ability of the microsomes, which can be detected even during the 1st week of feeding of 3'-methyl-4-dimethylaminoazobenzene, reaches a minimum at 4 weeks. If the feeding of the dye is continued, the swelling ability gradually returns to a nearly normal level at about 20 weeks. The swelling properties of microsomes from hepatomas which are induced by

3'-methyl-4-dimethylaminoazobenzene, resemble those from the liver of rats after the dye has been fed for 4 weeks. These findings suggested that after 4 weeks a critical period is reached in which a few surviving cells undergo certain irreversible structural alterations and become tumor cells. In contrast to these findings with the carcinogen, no appreciable change in microsomal swelling could be observed when animals were fed the non-carcinogenic isomer, 2-methyl-4-dimethylaminoazobenzene.

It was felt that a correlation between the changes observed in microsomal swelling and the actual appearance of gross tumors would provide an indication of whether these phenomena are

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involved in the carcinogenic process. For this reason the effect on the tumor incidence of feeding 3'-methyl-4-dimethylaminoazobenzene for various intervals has been studied. The evidence presented now supports the hypothesis that feeding this dye beyond the critical 4 weeks period does, in fact, cause the animals to reach a "point of no return," since the curve of tumor incidence as a function of the time of dye-feeding is not a straight line but shows a point of inflexion followed by a sudden steep rise at about 4 weeks.

A second phase of the present investigation was concerned with the problem of whether during chemical carcinogenesis primary cellular alterations that can be detected by the study of swelling are restricted to the endoplasmic reticulum, as was felt previously (1) and suggested later by the observations of Porter and Bruni (2), or whether the alterations occur in other cell particulates as well. Actually, it has been found that the swelling curves of liver mitochondria show essentially the same alterations at 4 weeks during feeding 3'-methyl-4-dimethylaminoazobenzene, as do the microsomes. As with these latter particulates, feeding of the non-carcinogenic 2-methyl-4-dimethylaminoazobenzene did not influence the swelling ability of the mitochondria either. The results imply that the endoplasmic reticulum is not an exclusive first target in the carcinogenic process, and are in agreement with previous reports (3, 4) that there are changes in the swelling properties of mitochondria during carcinogenesis.

In the third part of the present investigations the effect of variables and of various compounds of biological interest on the swelling of mitochondria was studied, with emphasis on the role of sulphydryl groups.

### Materials and Methods

#### Care and Feeding of Animals:

Sprague-Dawley male rats (Holtzman Rat Co., Madison, Wisconsin) with an initial weight of 180 to 230 gm., housed two in a cage, were fed *ad libitum* a semi-synthetic diet (5) (diet No. 2), called hereafter basal diet, identical to that previously used (1). The hepatic carcinogen 3'-methyl-4-dimethylaminoazobenzene, or the relatively non-carcinogenic 2-methyl-4-dimethylaminoazobenzene were incorporated in this diet to the extent of 0.06 per cent when the effects of these compounds were studied.

For the tumor incidence experiments, 9 groups of 16 to 17 rats per group were fed basal diet for 1 week, after which they were fed the diet containing 3'-methyl-

4-dimethylaminoazobenzene for periods varying from 1 to 12 weeks. Feeding of the basal diet was resumed after the respective periods of feeding the diet containing the dye. All animals were sacrificed a total of 7 months after initiation of the experiment. The macroscopically visible nodules on the livers were fixed in Bouin's fixative and examined histopathologically.

The percentage of tumor incidence in each group was calculated as the ratio of the number of tumor-bearing rats to the sum of all survivors plus those animals sacrificed earlier because of rapid tumor development. The rats having histopathologically benign hepatomas, 4 in the 5 weeks group, 3 in the 6 weeks group, and 2 in the 8 weeks group, have been included in these calculations as tumor bearers.

For the swelling experiments the control animals for the azo dye or tumor studies were fed basal diet for at least 2 weeks. The rats fed azo dyes were maintained previously on basal diet for 1 week. The rats used to study the effect of various compounds on mitochondrial swelling, and the corresponding control animals, were fed Purina laboratory chow.

#### Preparation of the Mitochondrial Fractions:

The rats were sacrificed by decapitation, the livers were perfused through the superior vena cava *in situ* with ice cold isotonic sodium chloride, and were excised. The firm white hepatomas which were used, were carefully dissected to eliminate the adhering liver tissue or any necrotic material. When liver tissues were used after prolonged feeding, all macroscopically visible nodules were eliminated before homogenization. All operations were carried out in the cold room.

The liver or tumor tissues were minced and then homogenized (20 per cent w/v. homogenate) in the cold in ice cold 0.44 M sucrose in a Potter-Elvehjem glass homogenizer using a teflon pestle. This sucrose solution also contained 0.001 M ethylenediaminetetraacetate (EDTA). Nuclei and cell debris were sedimented at 700 g, for 10 minutes, in the cold. The mitochondrial fraction, used in these experiments, was isolated from the supernatant fluid by centrifugation for 10 minutes at 13,000 g (2-3 °C.), according to Siekevitz and Watson (6). The resulting pellet was washed twice by resuspension and homogenization. EDTA was present in the 0.44 M sucrose used in the first washing but not in the sucrose used in the second washing. "Fluffy layer," if present, was removed. The final pellet was resuspended in 0.44 M sucrose so that 1 ml. contained the mitochondria from 1 gram of fresh tissue. This standard stock suspension, kept in ice, was always used within 10 minutes after preparation.

To study the effect of the feeding of 3'-methyl-4-

<sup>1</sup> The authors wish to thank Dr. Victor M. Arean and Dr. Joseph Simon for the histopathological examinations.

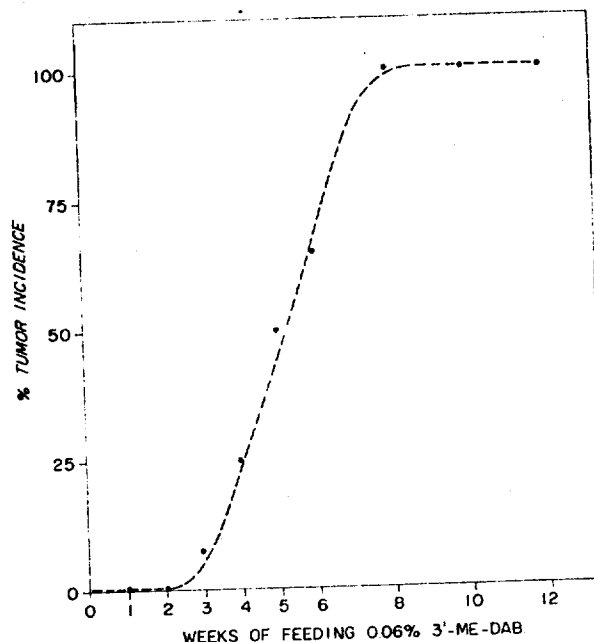


FIG. 1. Hepatic tumor incidence in 9 groups of rats 16 animals each (except 17 for 6 weeks), as a function of the time of feeding a semi-synthetic diet (1, 5) containing 0.06 per cent of 3'-methyl-4-dimethylaminoazobenzene. In all cases the tumor incidence was determined after 7 months.

dimethylaminoazobenzene and of the 2-methyl isomer on the amount of mitochondria present in the tissue, 10 gram samples of liver were used. The final mitochondrial suspensions were precipitated with trichloroacetic acid, washed as already described (1), dried, and weighed. These determinations were carried out in triplicate.

#### Swelling Tests:

(a) *By the Optical Density Method.*—Mitochondrial volume changes were followed at room temperature ( $\sim 25^\circ\text{C}$ .) by the optical density method introduced by Cleland (7), as modified by Tapley (8). The basic test system consisted of 4.7 ml. 0.3 M sucrose containing  $0.02\text{ M}$  tris(hydroxy-methyl-amino-methane) (Tris) buffer, 0.2 ml. of a stock solution containing thyroxine, calcium chloride, or mercuric chloride and that volume (0.05 to 0.2 ml.) of mitochondrial standard stock suspension which gave an initial optical density of 0.300. Since the percentage change in optical density during swelling of a given mitochondrial preparation depends to a certain extent on the initial optical density, all studies were made with test solutions with an initial optical density between 0.290 and 0.310. Furthermore, by conducting the assay in this manner, adjustment was automatically made for changes in the concentration of liver mitochondria during feeding of azo dyes. The final concentrations of thyroxine, calcium chloride, and mercuric chloride in the photometer cells were

$1 \times 10^{-5}\text{ M/l}$ ,  $5 \times 10^{-3}\text{ M/l}$ , and  $1 \times 10^{-5}\text{ M/l}$ , respectively. Many experiments were also conducted with 0.17 M sucrose which contained the same amount of Tris buffer but was used without added thyroxine, calcium chloride, or mercuric chloride. Here also 5 ml. was the final volume of the test system. The pH of the buffered 0.30 M and of the 0.17 M sucrose solutions was 7.4, except when the pH dependence of mitochondrial swelling was studied. The mitochondrial stock suspension was always added last to the test system. The decrease of optical density was followed at 520 m $\mu$  in rectangular absorption cells. The first reading was taken within 10 seconds after addition, and subsequent readings at 5 or 10 minute intervals for 40 minutes. The decrements of optical density were recalculated in "percentage swelling" as previously described (1). All swelling curves were determined in duplicate or triplicate, using mitochondrial fractions of individual rats. The data presented under "Results" in Figs. 2 *a* and *b*, 3 *a*, *b*, and *c*, and in Table I are based on the final state (per cent swelling) at 40 minutes. Since the comparison of the individual swelling curves (showing the rate of swelling) does not give, in this case, different or additional information, the above presentation has been used for the sake of clarity.

The unused portions of the mitochondrial stock suspension of the rats fed 3'-methyl 4-dimethylaminoazobenzene were precipitated with trichloroacetic acid.

## FINE STRUCTURAL ALTERATIONS IN CELL PARTICLES

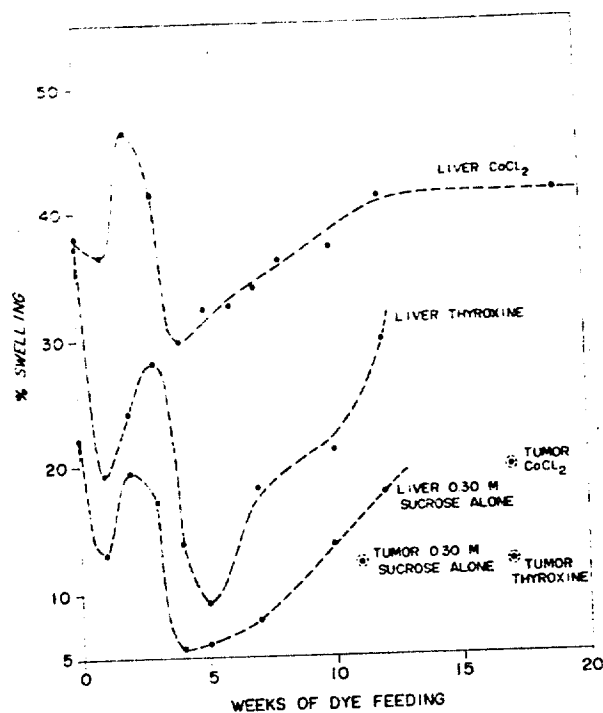


Fig. 2 a

FIGS. 2 a and b. Swelling of rat liver mitochondria in 0.30 M and 0.17 M sucrose containing Tris buffer (pH 7.4) as a function of the time of feeding 0.06 per cent 3'-methyl-4-dimethylaminoazobenzene. The agents used to induce swelling, calcium chloride, thyroxine, and mercuric chloride, were always employed in 0.30 M sucrose, and their respective concentrations were  $5 \times 10^{-3}$  M,  $1 \times 10^{-3}$  M and  $1 \times 10^{-5}$  M. The 0.17 M sucrose was used without added inducer of swelling. Each point of these curves corresponds to the swelling at 40 minutes. Lead nitrate at  $1 \times 10^{-5}$  M was found to produce a 90 per cent inhibition of the swelling of normal rat liver mitochondria, while cupric nitrate gives only a very slight inhibition of swelling even at  $1 \times 10^{-2}$  M.

washed as described (1), and resuspended in 3 per cent trichloroacetic acid for direct observation of the bound dye.

The compounds tested for possible action on swelling were either directly dissolved in the pH 7.4 sucrose buffer solution or added to the test system as small aliquots (0.1 to 0.2 ml.) of a concentrated stock solution adjusted to pH 7.4. The N2H mustard (Merck mustard) solution was not neutralized to avoid hydrolysis and was the only compound which was added to the test system after the mitochondrial stock suspension.

The optical densities were always read against a blank of distilled water, except for tannic acid, bilirubin, and biliverdin, in which the blank consisted of the same complete test system but without added mitochondrial stock suspension.

(b) By Gravimetry: The change in the swelling of mitochondria during feeding of 0.06 per cent 3'-methyl-4-dimethylaminoazobenzene was followed also by determination of the percentage dry weight of mito-

chondrial pellets. The method used was essentially that of Price, Fennesu, and Davies (9).

The final mitochondrial pellets obtained from 10 gram tissue samples (in tared centrifuge tubes) were resuspended in the same tubes in 0.17 M Tris buffered pH 7.4 sucrose solution, and incubated for 20 minutes at room temperature. The swollen mitochondria were sedimented as usual and the weight of the wet pellets determined after the outer and inner walls of the tubes had been carefully wiped with "celluwipe." The tubes containing the pellets were then dried overnight at 90 °C., placed in desiccator and weighed after cooling. These experiments were carried out in duplicate.

## RESULTS

#### Tumor Incidence as a Function of the Time of Feeding 3'-Methyl-4-Dimethylaminoazobenzene:

Fig. 1 shows that a sudden steep rise in the hepatic tumor incidence in groups of rats fed under our experimental conditions 3'-methyl-4-



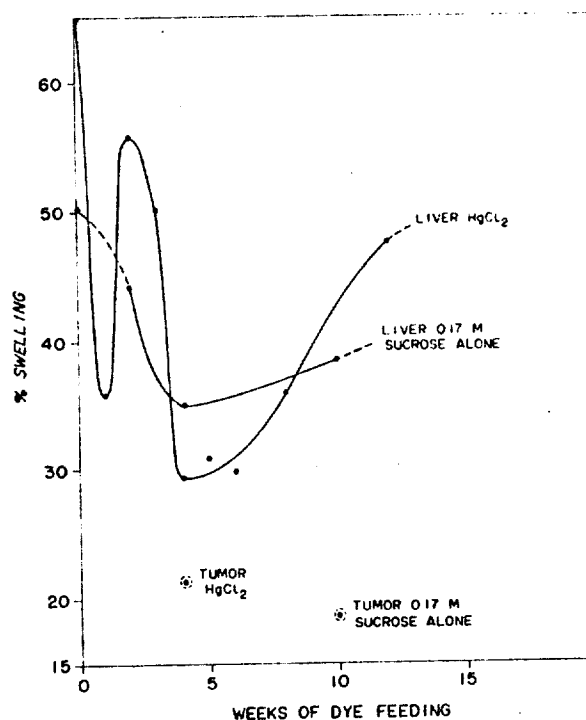


FIG. 2 b

dimethylaminoazobenzene for increasing time intervals, occurs at about 4 weeks. The ratios of the number of tumor-bearing rats over the effective totals have been given for most groups in a preliminary report (10). The curve is linear between 4 and 7 weeks and a plateau is reached at about 8 weeks. The curve shows that if the feeding of the dye is continued beyond 4 weeks, the animals rapidly reach a point of no return. The critical time coincides with the time of minimal microsomal and mitochondrial swelling.

The non-linearity of the curve near the origin is not surprising since several authors have already shown (11-13), that in order to induce liver tumors in rats with various agents, the carcinogen must be administered for a certain critical period, after which tumors may appear without further treatment. This period may vary with the experimental conditions and the nature of the carcinogen. It has been shown for one particular case that the end of this critical period coincides with minimum cellular levels of protein, pentose nucleic acid, and riboflavin (14), and with a sudden rapid cell proliferation (13).

The sudden sharp rise of the tumor incidence shown in Fig. 1 suggests that once beyond the

critical period, certain irreversible fine-structural alterations may become rapidly established in some liver cells by further feeding of the carcinogen. This aspect of the malignant transformation recalls the breakdown in the cell of the steric conformation of highly organized macromolecular regions, which is akin to a first order phase-transition of oriented polymer molecules (15, 16) in the sense described in detail by Flory (15). This phenomenon can be compared to the partial breakdown of simple crystal lattices; e.g., during thawing or irradiation. Following statistical thermodynamics the entropy of a system is proportional to the number of possible conformations of its constituting units. Thus, the loss of preferential structural orientations, that is, from ordered toward random alterations of highly organized macromolecular regions, corresponds to the increase of the entropy of the cell considered as a closed system. Similar conclusions were reached earlier by Ambrose (17) on the basis of the comparison of histological patterns, and by Rondoni (reviewed in reference 18) who, on correlating various experimental data, observed similarities between the process of carcinogenesis and protein denaturation.

*Mitochondrial Swelling during Feeding of Aminoazo Dyes:*

Figs. 2 *a* and *b* show the changes in the swelling ability of rat liver mitochondria (determined by the optical density method), as a function of the time of feeding 3'-methyl-4-dimethylaminoazobenzene. These figures complete the data given in a preliminary report (10). As with the microsomes (1), all of these curves show a minimum level at 4 weeks (except for thyroxine at 5 weeks) followed by a phase of apparent recovery. The swelling of mitochondria from hepatoma is generally comparable to or lower than the swelling of these same particulates from liver at the minimum point of the curve, in the same experimental conditions. Thus, the mitochondria from hepatoma seem to be very resistant to swelling (*cf.* 3, 4).

The change in the swelling ability of the mitochondria as a result of feeding this azo dye was similar when determined by the percentage dry weight of the mitochondrial pellet. The swelling after 20 minutes in 0.17 M sucrose containing Tris buffer (pH 7.4) was measured by this method on mitochondria isolated from the livers of normal rats, from rats fed 0.06 per cent 3'-methyl-4-dimethylaminoazobenzene for 2, 4, 6, 8 and 10 weeks, and from hepatoma. As with the optical density method, there was a minimum in the swelling at 4 weeks, which was then followed by a recovery phase. Very little swelling was observed with the mitochondria from hepatoma.

As with the microsomes (1), feeding at the same level of the relatively non-carcinogenic 2-methyl-4-dimethylaminoazobenzene did not seem to influence noticeably mitochondrial swelling even after 6 weeks, as shown in Table I.

Thus, mitochondrial swelling shows essentially the same alterations during azo-dye carcinogenesis as does microsomal swelling.

The only noticeable feature of the mitochondrial swelling curves, which could not be observed with the microsomes (1), is that there is an early recovery phase of the swelling with a maximum at 2 weeks. It may not be excluded that there may be a relation between this early recovery phase of swelling and the time of maximum dye-binding at about 2 weeks in these particles. That the protein-bound azo dyes first observed by Miller and Miller (19) are distributed all over the cell (20-22), is now well established. Quantitative studies have shown already that the maximum binding of 3'-methyl-4-dimethylaminoazobenzene by microsomes occurs between 2 and 3 weeks (1), as with

TABLE I

*Swelling of Liver Mitochondria of Rats Fed 0.06 Per Cent 2-Methyl-4-Dimethylaminoazobenzene*

Same experimental conditions as in legend of Figs. 2 *a* and *b*.

	Normal	2 weeks	4 weeks	6 weeks
$5 \times 10^{-3}$ M $\text{CaCl}_2$	40.8	36.7	42.8	36.3
$1 \times 10^{-5}$ M $\text{HgCl}_2$	65.1	65.5	62.4	65.8

the total liver (19). Although these quantitative studies (1) could not be extended yet to the mitochondria, observation of the trichloroacetic precipitates of the mitochondrial fractions in 3 per cent trichloroacetic acid (see Swelling Tests under Materials and Methods) clearly shows that as with microsomes the maximum level of dye binding to mitochondria occurs between 2 and 3 weeks. This early recovery phase of the swelling at 2 weeks suggests a response of the mitochondrial fine-structure, compensating for the loss at 1 week of the initial swelling ability of these particulates. In contrast, the *late* recovery of the swelling that takes place beyond the critical 4 weeks is likely to be of a different nature. In fact, this *late* recovery of the swelling coincides in time with the large and rapid decrease of the bound 3'-methyl-4-dimethylaminoazobenzene, in spite of the continuous feeding of this carcinogen. In this connection, the existence in the early phase of azo dye feeding of other compensatory responses in the soluble cytoplasmic proteins may be suggested by the work of Sorof *et al.* (23).

*The pH Dependence of Mitochondrial Swelling:*

The important differences between the macromolecular organization of liver mitochondria and of hepatoma mitochondria are indicated not only by changes in the extent of swelling, but also by drastic alteration of the pH dependence curves when passing from the liver to the hepatoma (Figs. 3 *a*, *b*, and *c*). Significant differences were also found previously with the microsomes (1).

All curves obtained with the liver mitochondria have relatively sharp maxima at pH = 7.5-8.0 (*cf.* 24, 25), while the mitochondria from hepatoma showed always much less swelling and gave rather flat pH dependence curves with no clear cut maxima. The pH dependence curve with calcium chloride of liver mitochondria shows a sharp minimum at pH = 9.0, which could not be seen in any other case. A difference can be observed

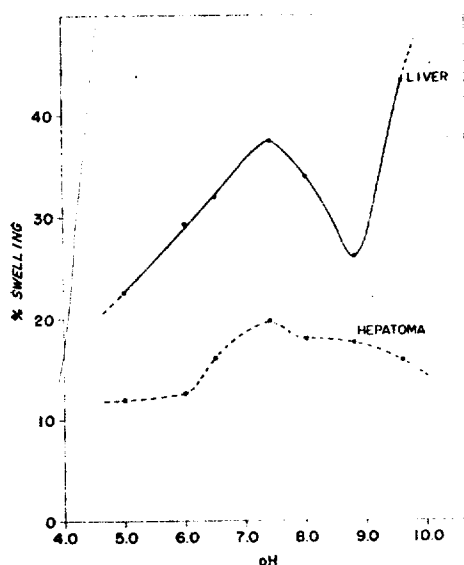


Fig. 3a

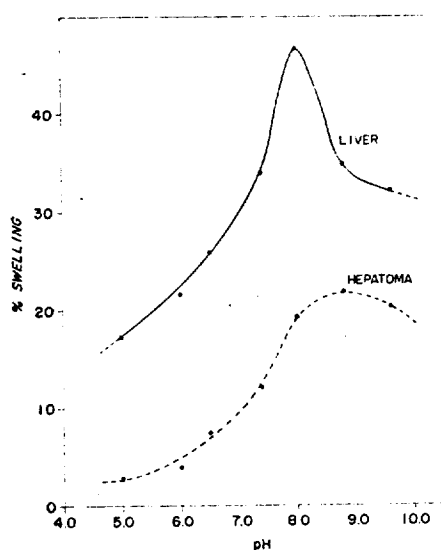


Fig. 3b

between the positions of the maxima of the liver (pH = 8.0) and of the hepatoma (pH = 8.8) when thyroxine is used as inducer.

*Influence of the Feeding of Azo Dyes on the Amount of Mitochondria in the Liver:*

Although Price, Miller, and Miller (20) have shown that there is a significant decrease in the mass of mitochondria which can be isolated from the livers of rats fed various carcinogenic azo dyes

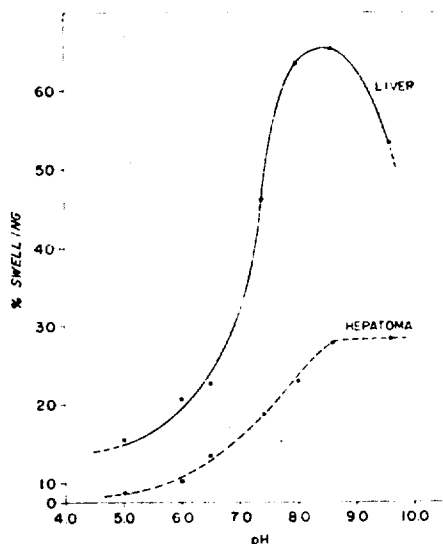


Fig. 3c

Figs. 3a, b, and c. Effect of pH on the swelling of mitochondria from liver and hepatoma of the rat. Fig. 3a in 0.30 M sucrose containing Tris buffer and  $5 \times 10^{-3}$  M  $\text{CaCl}_2$ ; Fig. 3b, the same sucrose, but with  $1 \times 10^{-5}$  M thyroxine; Fig. 3c in 0.17 M sucrose containing Tris buffer, without added inducer. All points on these curves correspond to the swelling at 40 minutes.

for 4 to 5 weeks, a study of the time course of this reduction has not been made. Moreover Schneider (26) and Schneider and Hogeboom (27) found that the mass of mitochondrial material which can be isolated from a hepatoma is much less than that isolated from the normal liver. In contrast to these findings, feeding of the relatively non-carcinogenic 2-methyl-4-dimethylaminoazobenzene causes a large increase in the amount of mitochondria in the liver (25). These variations probably correspond to changes in the number of mitochondria per cell (28).

The data presented in Fig. 4 constitute further confirmation of the previous observations (20, 28) and indicate that the diminution of the amount of mitochondria reaches a plateau after about 4 to 5 weeks. The results suggest that the recovery of the swelling of liver mitochondria after 4 weeks does not depend upon a return to normal of the amount of material in this fraction. On the other hand, feeding the same level of 2-methyl-4-dimethylaminoazobenzene, produced a 67 per cent increase in the dry trichloroacetic precipitate after 2 weeks and 86 per cent after 6 weeks, in agreement with previous results (20).

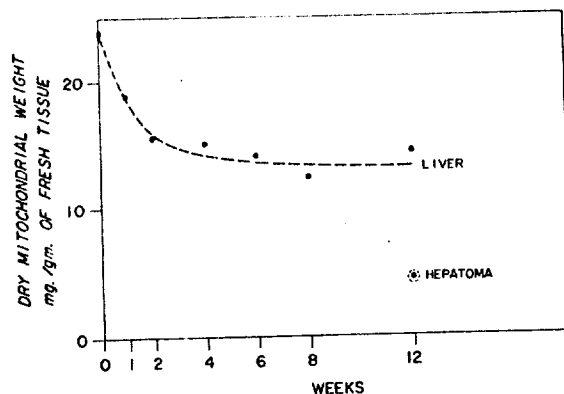


FIG. 4. Variation of the dry weight of the trichloroacetic acid precipitated mitochondrial fraction as a function of the time of feeding 0.06 per cent 3'-methyl-4-dimethylaminoazobenzene.

#### *Influence of Various Compounds on Mitochondrial Swelling:*

**Keto Compounds.**—Carbonyl groups of various organic compounds are known to interact with proteins. For example, the diabetogenic action of alloxan (29) and glyoxal (30) may well result from the irreversible blocking of critical sulfhydryl groups of the beta cells (31).

Consequently, we felt that the study of the effect of carbonyl compounds on mitochondrial swelling may shed some light on the steric correlation or the relative distance of the sulfhydryl groups which seem to play an important role in the permeability of the mitochondrial membrane (8).

Fig. 5 shows that at least two adjacent carbonyl groups are required for the inhibition of mitochondrial swelling, since at the same molar concentration only alloxan and diacetyl are active, while acetone, chloroacetone, acetylacetone, and acetylacetonone are inactive.

Tapley reported (8) that another sulfhydryl reagent, iodoacetamide, at  $1 \times 10^{-2}$  M is as potent an enhancer of swelling as mercuric chloride at  $1 \times 10^{-5}$  M. However, in our experiments, fluoroacetamide or sodium fluoride at various concentrations from  $1 \times 10^{-5}$  M to  $1 \times 10^{-2}$  M had no effect on mitochondrial swelling.

The effect of some of these compounds on the swelling of the mitochondria of livers from rats fed 3'-methyl-4-dimethylaminoazobenzene was also studied. It was found that the inhibition of swelling caused by diacetyl is abolished when 0.06 per cent 3'-methyl-4-dimethylaminoazobenzene is fed for 10 weeks, but not when it is fed for

only 2 or 4 weeks. On the other hand, the absence of inhibition by acetylacetone and acetylacetonone is not affected by feeding this dye for the same period of time.

**Sulfhydryl Compounds.**—Fig. 6 shows the effect of three sulfhydryl compounds on mitochondrial swelling. The observation that reduced glutathione at  $1 \times 10^{-2}$  M causes considerable enhancement of swelling confirms the recent data of Lehninger and Schneider (32). Freshly prepared sodium thiosulfate or sodium hydrosulfite, however, did not affect swelling at  $1 \times 10^{-2}$  M or  $1 \times 10^{-3}$  M. Another sulfhydryl agent, the 2, 3-dimercaptopropanol (BAL) caused nearly total inhibition at  $1 \times 10^{-2}$  M.

The slight enhancement of swelling caused by  $\alpha$ -lipoic acid (6,8-dithio-octanoic acid) cannot be attributed to the surface active properties of this compound, since the unsubstituted *n*-octanoic acid causes total inhibition of swelling at the same concentration. This finding on the effect of  $\alpha$ -lipoic acid is consistent with the observation of Biesele (33) that mouse fibroblasts treated with this same agent in tissue culture show somewhat enlarged mitochondria.

The effect on mitochondrial swelling of a large number of compounds of other classes has also been tested, such as: cross-linking and surface active agents, narcotics, antibiotics, substituted anilines and phenols, purine analogs, and derivatives; moreover, diphosphopyridinenucleotide (DPN), histamine, bilirubin, biliverdine, heparin, and alginic acid. These swelling tests have also been carried out in the 0.17 M sucrose assay system. The whole spectrum of the possible effects

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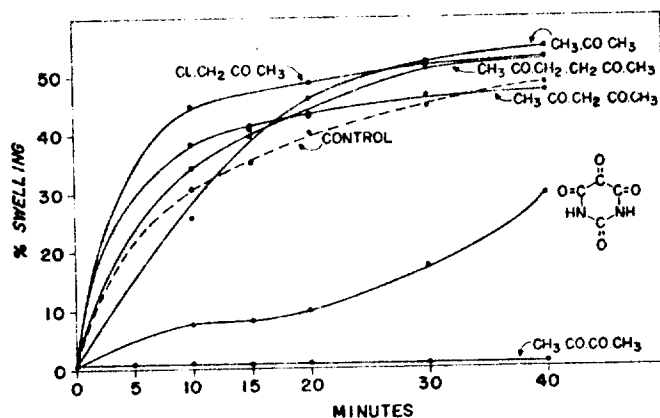


FIG. 5. Effect of keto compounds on the swelling of rat liver mitochondria in 0.17 M sucrose, containing Tris buffer, pH 7.4. All compounds were at  $1 \times 10^{-2}$  M. Diacetyl, acetylacetone, acetonylacetone, and chloroacetone were freshly tri-distilled samples. At  $1 \times 10^{-3}$  M, alloxan caused 45 per cent enhancement of swelling.

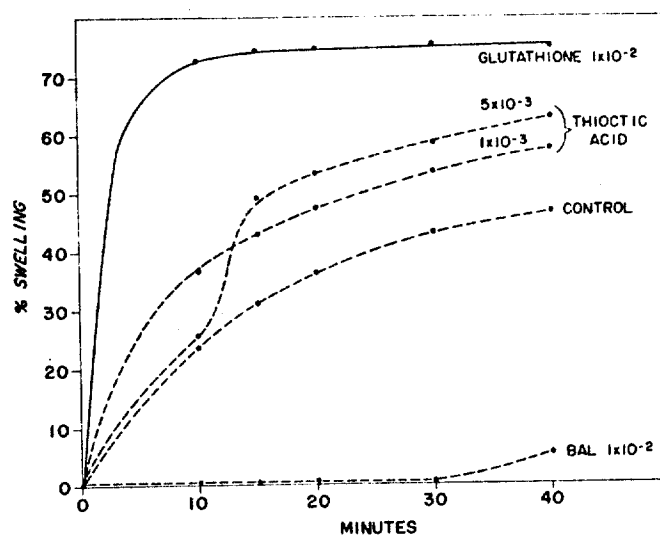


FIG. 6. Effect of sulfhydryl compounds on the swelling of rat liver mitochondria, conditions as in Fig. 5. 2,3-Dimercaptopropanol had no effect on swelling at  $1 \times 10^{-3}$  and  $1 \times 10^{-4}$  M, and glutathione at  $1 \times 10^{-3}$  M.

on mitochondrial swelling has been observed in these tests. The only consistent pattern of the mechanism of action that emerges from these tests is that the cross-linking agents, the surface active agents at lower concentrations, and the substituted anilines having an electronegative group in para position inhibit mitochondrial swelling, possibly through two- or multipoint attachment such as in the model proposed for *p*-aminobenzoic acid (34). It is also noteworthy that under our experimental conditions  $5 \times 10^{-3}$

M DPN caused considerable enhancement of swelling and biliverdine gave strong inhibition at  $5 \times 10^{-4}$  M, while the presence of the same concentration of bilirubin resulted in a marked aggregation of the mitochondria.

The fact that various compounds can affect the swelling so differently suggests that it is not the mere presence of specific functional groups that are the determining factors, but rather the general structural pattern of the whole molecule. This, in turn, may indicate that the various structurally

unrelated chemical agents that affect mitochondrial swelling, act in the membrane structure possibly on different macromolecular sites (*cf.* reference 24).

#### DISCUSSION

Evidence has been available to the effect that there are changes of the macromolecular organization of rat liver mitochondria during chemical carcinogenesis. Clerici and Cudkovicz (3) have observed by means of electron microscopy an increase of rigidity as a consequence of feeding 4 dimethylaminoazobenzene. Emmelot and Bos reported that the swelling of rat liver mitochondria, induced by thyroxine, decreases after this same dye has been fed for 5 months (4), or by incubating normal mitochondria *in vitro* with the carcinogens 4-monomethylaminoazobenzene or *o*-aminoazotoluene (35). However, no stepwise time study for the successive changes has been reported, such as described for the microsomes (1).

The present work establishes that these modifications of the mitochondrial swelling occur *simultaneously* with similar alterations undergone by the microsomes. The alterations that can be detected by the study of swelling in the microsomal and mitochondrial fine structure seem to be directly involved in the carcinogenic process since (a) these alterations cannot be produced in either particles by feeding a non-carcinogenic azo dye of similar structure<sup>2</sup> and (b) there is a close correlation between the minimal feeding period required for the appearance of gross tumors and the time of minimum swelling in these two types of particles.

The present results suggest that the carcinogenic process does not start, as was felt previously (1), within a single type of cellular component but rather occurs at more than one subcellular locus. The result of the structural alterations of the subcellular particles may well be that the rates of flow of nucleotides, various organic cofactors, and inorganic ions among the cell components (36) are modified, thereby introducing disturbances in various feedback mechanisms which may possibly control the rhythm of cell division (37).

One of the simplest explanations of the higher structural rigidity of the tumor mitochondria as compared to that of the liver mitochondria is that

the membrane of the former has a more extensively cross-linked lipoprotein structure. Since disulfide bonds are undoubtedly the most common covalent bonds cross-linking the polypeptide chains in proteins (*e.g.* 38), it is reasonable to suppose that one of the structural bases of greater membrane rigidity may be the increase of the cystine content of these organelles during carcinogenesis. *In fact*, it has been reported (39) that mitochondria of the rat hepatoma contain 44 per cent more cystine than the mitochondria of the rat liver.

Although the writers feel that the above explanation constitutes the most likely mechanism of the observed increase in structural rigidity, the possible role of other factors may not be disregarded, such as *e.g.*: (a) change in the character of liver cell population because of extensive bile-duct proliferation in the early periods of feeding (40); (b) the possible decrease in the availability of oxidizable substrate necessary for mitochondrial swelling (*e.g.* reference 41); and (c) depression of the level of DPN (42) possibly influencing the state of oxygenation of the respiratory carrier. It should be added, however, that the phenomena (b) and (c) may be, in fact, the consequences of structural alteration of the mitochondrial membrane, respectively through decreasing the steric availability of substrate at critical sites, and by altering the protein carrier to which DPN is bound. Views similar to the latter were expressed earlier by Greenstein in relation to the low B vitamin levels in tumor tissues (43).

*In conclusion* the following mechanism is tentatively suggested for the action of azo dyes at the level of the mitochondrial membrane:

The azo carcinogens act at this level by inhibiting swelling (*cf.* 3, 4, 35) possibly by cross-linking macromolecular elements that can undergo reversible structural changes and, thus, condition the dynamic behavior of the mitochondrial membrane. That, in the conjugated aromatic carcinogens, the 4 and 4' (or equivalent) positions are the points of interaction, has been suggested first under the term of "para principle" by Druckrey (44) and later by Buu-Hoi (45), and extensively discussed on the basis of physicochemical considerations by one of us (46). An important evidence that can be cited in favor of this concept is the observation of Cheesman (47) that the elasticity of monomolecular layers of globin (as measured by the pressure-area relationship) is strongly depressed by derivatives of the carcinogenic

<sup>2</sup> Preliminary results of experiments conducted in our Laboratory with several other agents: two carcinogenic and two non carcinogenic azo dyes, 2-acetylaminofluorene, ethionine and tannic acid, are consistent with the findings described in the present report.

4-dimethylaminostilbene, a close structural analog of the azo dyes. The considerable enhancement of the carcinogenic activity of 4-dimethylaminoazobenzene by introduction in 4' of certain substituents (such as  $-F$ ,  $-C_2H_5$ , or  $-N \rightarrow O$  as pyridine *N*-oxide in the ring), which can establish interactions by the various types of secondary valence forces, constitutes another supporting evidence.<sup>3</sup>

The structural rigidity of the mitochondrial membrane, which is maintained in the early periods of feeding by the continuous presence of the cross-linking carcinogens, may become, beyond a critical period of administration, an irreversible characteristic of a few cells. Once established in the form of a new macromolecular pattern, this new property may be transmitted to subsequent generations of cells. Such observed continuity of an acquired extra nuclear property does not presuppose or imply, however, any specific mechanism of transmission of cellular characteristics.

Such drastic alterations in the membrane structure cannot fail to affect the enzymes of the respiratory chain for which it serves as an "embedding core" (48). Thus, by modification of the distribution of the enzymic sites (*cf.* 43) new or alternative metabolic pathways may be established or favored, channeling substrates and electrons in new directions. It is possible that the aerobic glycolysis coexisting with the high rate of respiration in the tumor tissue is the functional expression of these specific structural alterations of the mitochondrial membrane, in conformity with the views voiced years ago by Rondoni (reviewed in reference 18).

The authors are greatly indebted to Dr. Charles F. Crampton and Dr. James A. Olson for helpful criticism and discussion.

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<sup>3</sup> Recent results in our Laboratory have shown that by introducing an ethyl group in the 4' position, the inactive azo dyes, 2-methyl 4-dimethylaminoazobenzene and 4-diethylaminoazobenzene, are transformed to potent hepatic carcinogens (unpublished). Similar observation was made with the latter compound when an  $\equiv N \rightarrow O$  group was introduced in 4' as pyridine *N*-oxide (E. V. Brown, personal communication).

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Diacetyl

It exerts a somewhat weaker effect than acetylacetone, but basically its effect is exactly the same, from a qualitative standpoint, as that of acetylacetone. The metaphase chromosomes remain perhaps still longer and the parallel-separating anaphases are more frequent (numerous).

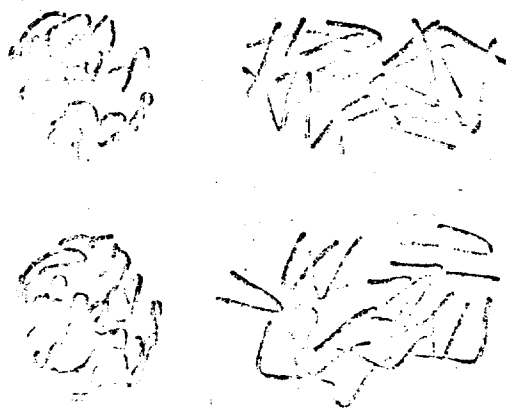


Fig. 11. Diacetyl 0.08-0.1 mol/l. On the left, two prometaphases, on the right two metaphases in typical formation (configuration): chromosomes have an irregular (random) distribution, but are not greatly shortened.

Protoplasma; 54: 455-475, (p. 469), 1962

## 25. Diacetyl (Tab. 2; Abb. 11)

Es entfaltet eine etwas schwächere, aber im wesentlichen qualitativ genau gleiche Wirkung wie Acetylaceton. Die Metaphasediosomen bleiben

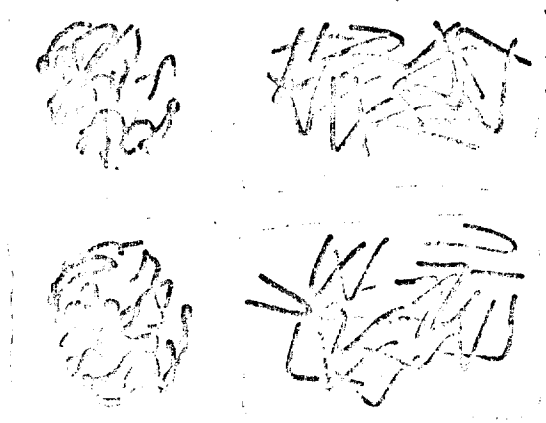


Abb. 11. Diacetyl, 0,08 - 0,1 mol/l. Links zwei Prometaphasen, rechts zwei Metaphasen in typischer Ausbildung: Chromosomen ungeordnet, aber wenig verkürzt.

ben vielleicht noch länger und die paralleltrennenden Anaphasen sind häufiger.

## 26. Benzophenon (Abb. 12)

Auch dieser einfachste Vertreter der aromatischen Ketone hat statismokinetischen Effekt, mit einem Optimum bei ungefähr 0,055 mol/l. Die Hemmung des Mitoseablaufs ist aber nicht so stark wie bei den aliphatischen

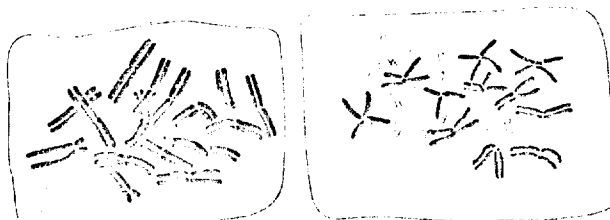


Abb. 12. Volle und späte Metaphase nach Behandlung mit 0,055 mol/l Benzophenon. Typische Statismokinese.

Diketonen, so daß paralleltrennende Anaphasen häufig zur Entwicklung kommen und während der Behandlung auch noch Restitutionskerne entstehen. Beim Vergleich mit Aceton ist auch hier wieder festzustellen, daß sich die statismokinetische Wirkung schon bei wesentlich niedriger Konzentration (0,05 mol/l) entfaltet als die merokinetische (1,0 mol/l bei Aceton).

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**Effect of Other Flavor Components on the Perception of  
Diacetyl in Fermented Dairy Products****SUMMARY**

The effect of fat, pH, and volatile acids on the perception of diacetyl was investigated. At the normal pH of skim milk or cream the threshold for diacetyl was 0.01 ppm. At pH 5.0 the threshold in skim milk was 0.2 ppm, and at pH 4.4 the diacetyl threshold in cream was 0.05 ppm. The fat content of cream probably suppresses the acid effect. The presence of propionic acid did not lower the threshold of diacetyl in sour cream. Formic acid lowered the threshold slightly, whereas acetic acid and acetaldehyde lowered it considerably.

**INTRODUCTION**

Diacetyl is an important contributor to the flavor of cultured dairy products. It is probably the major flavor component, although a number of other flavors are present (Parker and Elliker, 1953; Eakle, 1963). It has been studied in starter cultures (Mather and Babel, 1959), creamed cottage cheese (Parker and Elliker, 1953), cheddar cheese (Schurt and Price, 1949), and butter (Riel and Gibson, 1961).

The amount of diacetyl present in creamed cottage cheese on the retail market apparently varies considerably (Parker and Elliker, 1953; Mather and Babel, 1959). The amount of diacetyl in creamed cottage cheese can be standardized by using citrated whey starters (Lundstedt and Fogg, 1962) or *Lactococcus citrovorus* cultures (Mather and Babel, 1959), or adding starter distillate.

Little information is available on thresholds for diacetyl and on the effect of other flavor components on the perception of diacetyl. It has been noted that chemical diacetyl is harsh in flavor (Eakle, 1963). Information on thresholds should be useful in future consumer preference studies and in making correlations with chemical and chromatographic studies of flavor components in cultured dairy products. An investigation of this kind has been made into thresholds for flavoring components in wine (Berg *et al.*, 1955a,b,c; Hinreiner *et al.*, 1955). This

study was made to determine the effect of some other volatile components on the detection of diacetyl in fermented dairy products.

**MATERIALS AND METHODS**

Thresholds and minimum perceptible differences for diacetyl were established in skim milk and in cream. The effect on the threshold of pH, kind of acid, and presence of volatile acids was determined.

In these experiments the threshold was defined as that concentration of diacetyl which could be detected by the panel as a whole at the 5% level of significance when compared with a similar sample containing no diacetyl. Significance in paired comparisons was determined by referring to tables of Roessler *et al.* (1959). When the effect of pH or presence of volatile acids on diacetyl threshold was determined, the samples were identical except for the diacetyl in one sample. The minimum perceptible difference was also determined by paired comparisons. In this instance both samples contained diacetyl, the lower concentration being five times the previously determined threshold.

During a 1-hr tasting session, 16 paired comparisons were presented to the judges at 3-min intervals. Four pairs were replicated four times in a random order. The order of presentation within pairs was also randomized. Milk samples were presented in numbered test tubes, and cream samples in paper cups. Approximately 5 ml of milk or cream was tasted.

The judges were junior and senior students in the Foods and Nutrition Department. Seven judges with the lowest thresholds were chosen from a larger group of students after a three-week training period. As a training device, judges were told which sample contained diacetyl as they tasted the pairs. However, after the judges became familiar with the flavor of diacetyl, samples were not identified until the tasting was completed. As training progressed, smaller differences were detected. During the experiment, 3 or 4 one-hour sessions were usually required to determine a threshold or the minimum perceptible difference. An attempt was made to include two pairs of samples in a tasting session which could not be told apart and two pairs which could be correctly

identified at the 5% level of significance. Judges gave a larger total number of correct responses when they were confident of the answers for some pairs.

The milk and cream products used in the experiment were prepared in the following ways. Skim milk with a solids content of 8.5-9.2% was heated at 38°C under reduced pressure and cooled to 20°C. Lactic acid was added to adjust the pH, and the mixture was cooled to 4°C.

Cream was standardized to 19% fat with skim milk. It was heated under reduced pressure to 38°C in a water bath. After the addition of 1.18% stabilizer (obtained from Meyer-Blanke Co.) the cream was heated 20 min in a water bath at 165°F. A magnetic stirrer with teflon-covered rod was used to agitate the mixture while heating and cooling. For production of sour cream by acidification with glucono-delta-lactone, the cream was cooled to 30°C, glucono-delta-lactone was added in an amount (17%) based on % solids-not-fat in the cream, the mixture was incubated 3 hr at 30°C, and then cooled to 4°C. For preparation of sour cream acidified with lactic acid, the cream was cooled to 20°C, lactic acid was added to the cream with agitation to the desired pH, and the mixture was cooled to 4°C. For both methods, pH was controlled within  $\pm 0.1$  unit.

Stock solutions of chemical diacetyl and of formic, acetic, and propionic acids containing 100 ppm were prepared and stored at 4°C. A fresh solution of acetaldehyde at this concentration was prepared each day. Calculated amounts of these solutions were added quantitatively to a measured or weighed amount of milk or cream. Samples were mixed and stored in closed containers at 4°C until immediately before tasting.

### RESULTS

Thresholds of diacetyl in skim milk, in general, were higher as the pH was decreased (Table 1). About 20 times as much diacetyl was necessary for detection at pH 5.0 as at the normal pH of milk. Minimum perceptible differences also increased about 20 times as pH was lowered. Except at pH 6.0, a difference between concentrations of

about 5 times the threshold was required before the higher concentration of a pair could be detected a significant number of times. Apparently a small amount of acid (pH 6.0) sharpened the taste for diacetyl. The threshold at this pH was lower than at the normal pH for milk and a smaller difference between concentrations was necessary. Levels of pH below 5.0 were not investigated, since the skim milk coagulated and the texture and strong acid flavor made the taste disagreeable.

At normal pH, the diacetyl thresholds for skim milk and cream occurred at the same concentration. In previous work with a different taste panel, the threshold in homogenized milk was also 0.01 ppm. However, the minimum detectable difference in paired samples was smaller in cream than in milk at pH 6.8. Apparently the fat content has little effect on perception of diacetyl at normal pH. No indication of taste fatigue was detected during tasting 16 pairs of cream samples with a high fat content. A 3-min interval between pairs of samples seemed to be adequate to remove after-tastes.

The effect of lowering the pH of cream is not as great as would be expected from the results with skim milk. The thresholds and minimum perceptible differences in skim milk at pH 5.5 and in cream at pH 4.4 were identical. The fat content of the cream may depress the apparent acid taste and allow diacetyl to be perceived more easily.

The minimum perceptible difference of diacetyl is smaller in cream without stabilizer (Table 2). This result may be due to a change in the texture of cream with added stabilizer or to flavors from the stabilizer used.

The kind of acidifying agent used to lower the pH did not affect the threshold, but a smaller difference in diacetyl level could be

Table 1. Threshold concentrations and minimum perceptible differences of diacetyl in skim milk at several pH values.

pH	Threshold concentration (ppm)	Minimum perceptible difference between pairs		
		Lower concentration of pair (ppm)	Higher concentration of pair (ppm)	Difference (ppm)
6.8	.01	.05	.10	.05
6.0	.005	.025	.04	.015
5.5	.05	.25	.50	.25
5.0	.20	1.00	2.00	1.00

Table 2. Threshold concentrations and minimum perceptible differences of diacetyl in cream at normal and low pH.

pH	Additional ingredients	Threshold concentration (ppm)	Minimum perceptible difference between pairs		
			Lower concentration of pair (ppm)	Higher concentration of pair (ppm)	Difference (ppm)
6.8	None	.01	.05	.055	.005
6.8	Stabilizer	.01	.05	.07	.02
4.4	Lactic acid and stabilizer	.05	.25	.50	.25
4.4	Lactone and stabilizer	.05	.25	.375	.125

detected when glucono-delta-lactone was used than when lactic acid was the acidifying agent.

Formic, acetic, or propionic acid, or acetaldehyde, were added to lactone-acidified cream at amounts at or slightly above the threshold for these volatile constituents. Propionic acid apparently did not affect the perception of diacetyl although it probably contributed to background flavor. The other

Table 3. Thresholds of diacetyl in lactone-acidified sour cream with added volatile constituents.

Volatile constituent	Amount added (ppm)	Diacetyl threshold (ppm)
Formic acid	0.1	.01
Acetic acid	0.23	<.001
Propionic acid	0.1	.05
Acetaldehyde	0.1	<.001

volatiles tested did affect the perception of diacetyl. Acetic acid and acetaldehyde had a very strong effect and the diacetyl threshold could not be accurately established in sour cream containing these volatiles. Judges were able to detect diacetyl in samples with acetic acid or acetaldehyde a highly significant number of times at the lowest diacetyl concentrations tasted.

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# The Reduction of Diacetyl and Acetoin in *Aerobacter aerogenes*

## Evidence for One Enzyme Catalyzing Both Reactions

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The reduction of diacetyl and acetoin has been shown to be catalyzed by one enzyme in *Aerobacter aerogenes*. The ratio of the two activities remains constant during purification into homogeneity, and the two activities appear in the same amount when the growing cells are exposed to acetate.

Juni [1] showed in 1952 that acetoin (acetyl-methyl carbinol) is formed by a sequence of two enzymes in *Aerobacter aerogenes*, one catalyzing the formation of acetolactate from pyruvate, and another decarboxylating acetolactate to yield acetoin. These enzymes, the pH 6 acetolactate-forming enzyme ( $E_1$ ) and acetolactate decarboxylase ( $E_2$ ) have been purified and characterized [2–8].

Strecker and Harary [9] described in 1954 two enzyme systems from *A. aerogenes* and *Staphylococcus aureus*, one catalyzing the reversible oxidation by NAD of 2,3-butanediol to acetoin, and the other catalyzing an essentially irreversible reduction by NADH of diacetyl to acetoin. These enzymes, butyleneglycol dehydrogenase ( $E_3$ ) and diacetyl reductase ( $E_4$ ) were partially purified, and they suggested that these enzymes were different. The formation of acetoin and 2,3-butanediol is shown in Fig. 1.

This paper provides evidences that  $E_3$  and  $E_4$  activities are associated with the same enzyme in *A. aerogenes*. This is shown by purification and characterization of the enzyme.

### EXPERIMENTAL PROCEDURE

#### Reagents

NAD, NADH, streptomycin sulfate, and diacetyl were obtained from Sigma Chemical Company. DEAE-Sephadex A-50 and Sephadex G-100 were purchased from the Pharmacia Laboratories, and hydroxyapatite was obtained from the Bio-Rad Laboratories.

#### Spectrophotometric Assays

Conditions under which a linear response of velocity to enzyme concentration is obtainable at

**Enzymes.** 2,3-Butanediol : NAD oxidoreductase (EC 1.1.1.4); 2-acetolactate carboxy-lyase (EC 4.1.1.5).

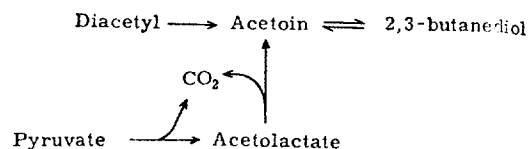


Fig. 1. Formation of acetoin and 2,3-butanediol from pyruvate in *A. aerogenes*

25°, for measurement of butyleneglycol dehydrogenase activity [9], consist, in a total volume of 3 ml, of the following amounts per ml: 0.1  $\mu$ mole NAD, 13  $\mu$ moles 2,3-butanediol, 50  $\mu$ moles phosphate pH 7.0 and enzyme. For measurement of diacetyl reductase activity, the 1 ml mixture contained 0.1  $\mu$ mole NADH, 13  $\mu$ moles diacetyl, 50  $\mu$ moles phosphate pH 7.5 and enzyme. A unit is defined as that amount of enzyme that gives a change in absorbance at 340 nm of 0.001 per min at 25°. A Beckman DB recording spectrophotometer was used for these assays. For stability of the enzyme upon dilution, see Results.

#### Protein

Protein was determined by the method of Lowry *et al.* [10] and for purified preparation with low protein concentration, by the absorbance at 280 nm. Protein bands in polyacrylamide gels were stained with 0.1% amidoblack in 5% acetic acid, 5% methyl alcohol, and water.

#### Buffers

The standard buffers, prepared at 20°, used throughout this work were the following: 50 mM phosphate containing 0.1 mM NAD and 25 mM 2-mercaptoethanol pH 7.0, and 50 mM Tris-Cl containing 0.1 mM NAD and 25 mM 2-mercaptoethanol

Table 1. Purification of the enzyme with butyleneglycol dehydrogenase ( $E_3$ ) and diacetyl reductase ( $E_4$ ) activities from *A. aerogenes*

Fraction and step	Volume	Protein	$10^{-3} \times$ Specific activity	Yield	Purification	$E_3/E_4$
	ml	mg	unit/mg	%	-fold	
I. Sonic extract from 36 g of cells	186	5022	44.5	100	1	0.30
II. Streptomycin sulfate	268					
III. $(\text{NH}_4)_2\text{SO}_4$	114	2964	64.6	86	1.5	0.29
IV. DEAE-Sephadex chromatography	100	180	862	70	19	0.31
V. Hydroxyapatite chromatography	89	39	2640	52	60	0.27
VI. DEAE-Sephadex chromatography	28	19	4800	32	107	0.25
VII. Sephadex G-100 chromatography	17	8	5530	20	124	0.27

pH 7.5. In the last two fractionation steps (DEAE and Sephadex chromatography) all buffers contained 20% glycerol. For stability of the enzyme, see Results.

#### Organisms

The organisms used throughout this work were wild type *A. aerogenes* strain 1033 and two mutants of *A. aerogenes* strain 35, IV-2 and 45-III, blocked in the formation of valine-isoleucine [11]. They were deficient in the pH 6 acetolactate-forming enzyme ( $E_1$ ) and butyleneglycol dehydrogenase ( $E_3$ ) activity, and mutant 45-III, in addition had a block in acetolactate decarboxylase ( $E_2$ ) activity. The bacteria were grown in minimal media as described [11,12], and strain 1033, for purification of the enzyme, was grown as reported [7].

### RESULTS

#### Purification of the Enzyme

The following procedure for purification of the enzyme associated with  $E_3$  and  $E_4$  activities resulted in a 124-fold purification with a 20% yield. Unless otherwise stated, all operations were performed at 0–5°, and Table 1 summarizes the data for a typical preparation.

#### Preparation of Extract

Frozen cells (36 g) were suspended in 5 volumes of the standard phosphate (see Experimental Procedure). The solution was subjected to ultrasound for 15 min with a Branson Sonic Power SW 75. The material was centrifuged for 30 min at  $40000 \times g$  and the clear solution was collected to yield Fraction I.

#### Streptomycin Precipitation

To the 186 ml of Fraction I were added, with stirring, 93 ml of a 10% streptomycin sulfate solution adjusted to pH 7.0 with phosphate. After standing for 15 min, the suspension was centrifuged and the supernatant collected (Fraction II).

#### Ammonium Sulfate Fractionation

To Fraction II were added, with stirring, 243 g per liter of solid  $(\text{NH}_4)_2\text{SO}_4$ , and the pH was adjusted to 7.0 by adding 1 M  $\text{K}_2\text{HPO}_4$  by drops. The suspension was allowed to stand for 20 min after the salt had dissolved. The precipitate was discarded after centrifugation at  $10000 \times g$  for 10 min, and to the solution were added 132 g per liter of solid  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was collected as described above, and dissolved in sufficient standard Tris-Cl (see Experimental Procedure) to provide a protein concentration of 20 mg per ml. This solution was then dialyzed against one liter of the same buffer overnight. A small precipitate, which appeared during dialysis, was removed by centrifugation at  $10000 \times g$  and yielded a clear supernatant (Fraction III).

#### DEAE-Sephadex A-50 Chromatography

Fraction III was loaded on the column (40  $\times$  3.5 cm) previously equilibrated with standard Tris-Cl (-NAD). The column was eluted with a linear NaCl gradient formed from 500 ml of standard Tris-Cl (-NAD), and of 0.5 M NaCl in standard Tris-Cl (-NAD). Fractions of 6.4 ml were collected at a flow rate of 60 ml per hour, and fractions 116 to 131 which contained the bulk of the activity, were collected (Fraction IV). Fig. 2.

#### Hydroxyapatite Chromatography

Fraction IV was made 0.1 M with NAD and was loaded on the column (8  $\times$  2.5 cm) previously equilibrated with 20 mM phosphate containing 25 mM 2-mercaptoethanol pH 7.0, at a flow rate of 30 ml per hour. The column was eluted with a 200 ml linear gradient between 20 and 300 mM phosphate pH 7.0 containing 25 mM 2-mercaptoethanol. Fractions of 6 ml were collected at a flow rate of 30 ml per hour, and fractions 39 to 53 were collected and made 0.1 M with NAD (Fraction V). 560 g of solid  $(\text{NH}_4)_2\text{SO}_4$  was added per liter to the pooled fractions and the suspension was centrifuged. The precipitate was dissolved in standard Tris-Cl and dialyzed against one liter of the same buffer.

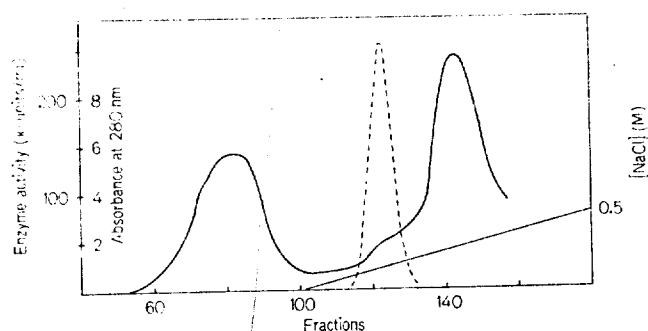


Fig. 2. Chromatography of Fraction III on DEAE-Sephadex A-50. A column,  $3.5 \times 40$  cm, was prepared and loaded with 114 ml of Fraction III. A constant linear gradient from 0 to 0.5 M NaCl in standard Tris-Cl (see Experimental Procedure), total volume of 500 ml, was applied at the flow rate of 60 ml per hour, and 6.4 ml fractions were collected. These fractions were assayed for butyleneglycol dehydrogenase ( $E_3$ ) activity (---), and their absorbance at 280 nm was determined (—).

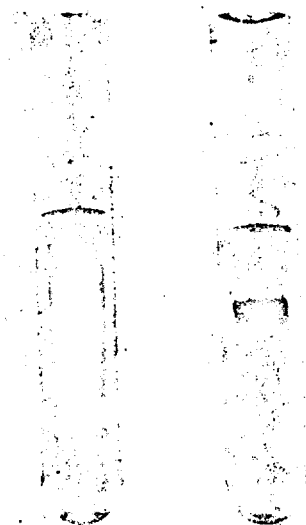


Fig. 3. Purity at stages in the purification of butylene-glycol dehydrogenase as monitored by polyacrylamide gel electrophoresis [13]. From left to right, the gels containing aliquots from Fractions VI, and VII. The direction of migration was toward the bottom of the gels (anode).

#### Second DEAE-Sephadex Fractionation

The dialyzed solution was loaded on the column and eluted as described above. The fractions were recombined (18–22) and solid  $(\text{NH}_4)_2\text{SO}_4$ , 560 g per liter, was added. The active precipitate was dissolved in standard phosphate (Fraction VI).

#### Sephadex G-100 Fractionation

Fraction VI was made 0.1 mM with NAD, and 560 g per liter of solid  $(\text{NH}_4)_2\text{SO}_4$  was added. The

Table 2. Effect of different organic acids on the formation of butyleneglycol dehydrogenase ( $E_3$ ) and diacetyl reductase ( $E_4$ ) activities

The cultures were incubated at  $37^\circ$  with gyrotory shaking, and the cells were harvested in their exponentially growing phase when the pH had dropped from 7.0 to 6.6, and resuspended in fresh medium pH 7.0. 40 ml portions were added on flasks containing 4 mmoles of the test compound previously adjusted to pH 7.0 with 1 M NaOH. The flasks were shaken at  $37^\circ$  for 60 min, rapidly cooled, and the cells were harvested and the specific activities of the enzymes determined.

Addition to media (100 mM)	Enzyme activities	
	$E_3$	$E_4$
	%	%
Acetate	100	100
Pyruvate	59	58
Formate	21	22
Propionate	21	19
Butyrate	16	16
Isobutyrate	20	14
Monochloroacetate	10	7
None	20	24

precipitate was dissolved in standard phosphate, volume 4 ml, and loaded on a column ( $20 \times 2.3$  cm) previously equilibrated with standard phosphate (-NAD). The column was eluted with the same buffer, and fractions of 4 ml were collected at a flow rate of 13 ml per hour. Fractions 9 to 12, which contained the purified enzyme (Fig. 3) were collected, and the total yield was 8 mg (Fraction VII).

#### Stability of the Enzyme

Storage of crude extract at  $0^\circ$  in phosphate pH 7.0 resulted in loss in activity (75% after one month). The inactivation occurred faster upon dilution or precipitation with ammonium sulfate, but could be prevented by the addition of 0.1 mM NAD and 10 mM 2-mercaptoethanol. When the enzyme was diluted, serum albumin (5 mg per ml) had to be present in order to prevent inactivation. A purified preparation has been stored for 6 months at  $0^\circ$  in the presence of these compounds and 20% glycerol, without significant loss in  $E_3$  and  $E_4$  activity.

#### Induction Studies

Evidence for coordinated induction of the 2,3-butanediol-forming enzymes ( $E_1$ ,  $E_2$ , and  $E_3$ ) in *A. aerogenes* by acetate, when grown on glucose, has recently been presented [12].

Table 2 shows the effect of various organic acids on the appearance of  $E_3$  and  $E_4$  in *A. aerogenes* strain 1033 and that acetate markedly stimulated the formation of the enzymes. The stimulation caused by pyruvate might be of secondary nature [12], since pyruvate can be converted to acetate.



Table 3. Effect of acetate on the formation of  $E_3$  and  $E_4$  activities  
For conditions, see Table 2

Strain	Enzyme activities			
	$E_3$		$E_4$	
	+	-	+	-
	100 mM acetate		100 mM acetate	
	%	%	%	%
1033	100	20	100	24
IV-2	17	3.0	17	3.2

When the cultures, in addition to acetate, contained 100  $\mu$ g per ml of *p*-fluoro-DL-phenylalanine, the specific activities of  $E_3$  and  $E_4$  were about 10% of that observed in the control.

Table 3 shows that mutant IV-2 has a partial block in  $E_3$  and  $E_4$ , with the same responses to acetate. Mutant 45-III which is blocked in  $E_1$ ,  $E_2$ , and  $E_3$ , showed negligible  $E_4$  activity.

#### DISCUSSION

The enzyme catalyzing the reduction of diacetyl ( $E_1$ ) and the reversible reaction between acetoin and 2,3-butanediol ( $E_3$ ) (Fig. 1) has been purified to homogeneity as judged by polyacrylamide gel electrophoresis. That these two reactions are catalyzed by one enzyme is, in addition, shown by a constant  $E_3/E_4$  ratio during purification. A 124-fold purification of the enzyme was obtained, and it represents about 0.8% of total protein in the cell. The pH 6 acetolactate-forming enzyme ( $E_1$ ) has been obtained in a 120-fold purification (0.8% of total protein) [2], and acetolactate decarboxylase ( $E_2$ ) in a 145-fold (0.7% of total protein) [8]. This indicates that the

three enzymes that convert pyruvate and diacetyl to acetoin and butanediol represent approximately 2.3% of total protein in the cell.

When the growing cultures were exposed to acetate, the amount of  $E_3$  and  $E_4$  increased in the same proportion. The results show that the effect of acetate is similar upon the formation of the butanediol-forming enzymes ( $E_1$ ,  $E_2$ , and  $E_3$ ) and diacetyl reductase ( $E_4$ ).

We suggest that the enzyme is named diacetyl (acetoin) reductase and a forthcoming paper will describe its physical properties.

We wish to thank Professor S. Laland at the Department of Biochemistry, University of Oslo, for providing facilities for this work.

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# CHR. HANSEN'S LABORATORY, INC.

9015 WEST MAPLE STREET  
MILWAUKEE, WISCONSIN 53214 • PHONE (414) 476-3630

September 14, 1973

Dr. Humphrey F. Saffoon  
Tracor-Jit Co., Inc.  
1300 E. Gude Dr.  
Rockwell, Md. 20851

Dear Sr. Saffoon:

Enclosed is a list of manufacturers who offer butter flavor intensifiers under which starter distillate is listed. You will note that there are quite a few.

As I mentioned earlier, to our knowledge we know of no other company who is producing a natural steam-distilled product such as ours. You will note there are several companies listed who offer this type of flavor component. We do know that Chumleas Laboratories produce this product which we think is a "synthetic". We also know that some of the other companies, i.e., Dairyland Foods, Marschall Division, Moseley Laboratories were at one time obtaining their products from another supplier. However, it could be now that they are producing some of their own.

I hope this list will be of some assistance. I regret I cannot give you more details on the individual companies.

I know your assignment concerning the Standard of Identify of this product has not been easy. I am sure, however, that you and your committee have exercised good judgment. If we can be of any further assistance, please feel free to call on us.

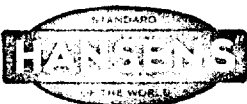
With best regards,

Sincerely yours,  
CHR. HANSEN'S LABORATORY, INC.



Robert L. Sellars, Ph.D., Vice President  
The Culture Research Center

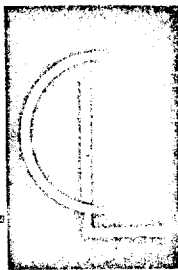
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# Chumlea's LABORATORIES

103 RYAN STREET • LEBANON, INDIANA 46052

*Manufacturers and Distributors of:*

Chumlea's DISTILLED BUTTER STARTER  
COTTAGE CHEESE COAGULATOR  
BUTTERMILK AND COTTAGE CHEESE FLAVOR

September 26, 1973

Dr. Humphrey Sassoon  
Tracor Jitco Inc.  
1300 East Gude Drive  
Rockville, Maryland 20851

Dear Dr. Humphrey:

We received your letter of September 19, 1973 regarding the identity of Starter Distillate.

We believe that the identification that you propose covers the product very satisfactorily.

As to safety as a food additive Hammer and Sherwood, Iowa Agr. Exp. Station research bulletin No. 8, 1923 report, that the main flavor ingredients in butter culture are Diacetyl Acetic acid and Propionic Acid.

These are the volatile Acids which are the main ingredients of Starter Distillate and are found in all cheese and cultural dairy products.

The recommended use of Starter Distillate in butter will amount to one (1) part diacetyl to 1,600,00 parts butter.

LMC/rp

Yours truly  
CHUMLEA'S LABORATORIES

*Leon W. Chumlea*  
LEON W. CHUMLEA, President

## Acute and Short-term Toxicity of Diacetyl in Rats

J. COLLEY\* and I. F. GAUNT—*Toxicology*

A. B. G. LANSDOWN and P. GRASSO—*Pathology*

and

S. D. GANGOLLI—*Analytical Chemistry*

*British Industrial Biological Research Association, Woodmansterne Road,  
Carshalton, Surrey, England*

(Received 10 April 1969)

**Abstract**—The acute oral and intraperitoneal  $LD_{50}$ s of diacetyl in rats lie between 3.0 and 3.4 g/kg and between 0.40 and 0.65 g/kg, respectively.

Rats were given 0 (control), 10, 30, 90 or 540 mg diacetyl/kg/day by oral intubation for 90 days. No adverse effects were found at the three lowest dosage levels. The highest dosage level caused a decrease in weight gain, an increase in water consumption, anaemia, an increased leucocyte count and an increase in the relative weights of the liver, kidneys and adrenal and pituitary glands. Stomach lesions seen at autopsy showed necrosis with infiltration by inflammatory cells.

The no-effect level (90 mg/kg/day) is equivalent to about 500 times the estimated daily intake by man (0.17 mg/kg).

## INTRODUCTION

Diacetyl (2,3-butanedione) is used to flavour foods, particularly baked goods, sweets and ice cream. It is formed in milk during the 'ripening' procedure that precedes butter or margarine production. Diacetyl is now added to fully ripened milk to augment the natural aroma, the final content being 1-3 ppm (Andersen & Williams, 1965).

The use of flavourings in the UK has been reviewed by the Food Standards Committee (1965), but so far no specific regulations to control their use have been produced. Diacetyl is permitted for use in foodstuffs in a number of countries including the USA, France, Belgium, Norway and Sweden.

Ketones are generally reduced to the corresponding secondary alcohol in the mammalian body, and these are eliminated following conjugation with glucuronic acid (Williams, 1959). The metabolism of diacetyl, however, has not been directly investigated. Jenner, Hagan, Taylor, Cook & Fitzhugh (1964) found that the acute oral  $LD_{50}$  of diacetyl was 1580 mg/kg in rats and 990 mg/kg in guinea pigs, but no other toxicological data are available.

\* Present address: Institute of Experimental Pathology and Toxicology, The Albany Medical College of Union University, Albany, New York 12208, USA.

The results of acute and short-term studies on diacetyl, carried out as part of the BIBRA safety evaluation programme, are reported here.

## EXPERIMENTAL

**Materials.** Diacetyl, supplied by Bush Boake Allen Ltd., London, complied with the following specification: Purity, 97%; relative density, 0.975-0.990 at 25°C; refractive index, 1.393-1.397 at 20°C; solidification point, -1.0 to -4.0°C.

**Animals and diet.** Both sexes of SPF-derived CFE rats were used in these studies. Spillers' Laboratory Small Animal Diet and water were provided *ad lib*.

**Loss of diacetyl from diet and water.** Diacetyl is highly volatile, and 88% was lost from a powdered diet over 48 hr. There was no loss from a 10% solution in water after 7 days exposure, but when rats were given a choice of water or 1% diacetyl to drink, the average daily consumption was 26.4 ml water but only 1.2 ml diacetyl solution. Because of the unpalatable nature of diacetyl solution and the loss of the compound from the diet, intubation was chosen as the method of oral administration. Analyses of diacetyl were carried out by gas chromatography on a Pye 104 dual flame ionisation chromatograph using a 5-ft glass column packed with Porapak Q at a temperature of 180°C.

## Experimental design and conduct

**Acute toxicity in rats.** Single doses of diacetyl were administered to groups of five adult rats by oral intubation or by intraperitoneal injection of a 20% solution in water. The rats were fasted for 18 hr before oral dosing, and after treatment were observed for 14 days for signs of intoxication. Autopsies were carried out on selected animals at 14 days.

**Short-term study in rats.** Groups of 15 male and 15 female weanling rats, housed five to a cage, were given by oral intubation a daily dose of 5 ml/kg of a solution containing 0.0, 0.2, 0.6, 1.8 or 10.8% diacetyl in water, providing doses of 0 (control), 10, 30, 90 or 540 mg diacetyl/kg/day, for 90 days. Food and drinking water were given *ad lib*. Body weight, food intake and water consumption were recorded weekly. Haematological investigations were carried out during wk 7 on blood collected from the tail veins of eight animals of each sex from the control, 90 and 540 mg/kg/day groups, and terminally on blood collected from the aorta of all animals. The blood was examined for haemoglobin content, packed cell volume and erythrocyte, reticulocyte and total differential leucocyte counts. Methaemoglobin levels were determined on all terminal samples. Liver function was examined terminally by measuring the activities of serum glutamic-oxalacetic and glutamic-pyruvic transaminases (Karmen, 1955). Kidney function was investigated at wk 7 on six animals of each sex from the control, 90 and 540 mg/kg groups and terminally on all animals. Urine was examined for colour, pH, microscopic constituents, glucose, bile salts, blood and activity of glutamic-oxalacetic transaminase. The blood urea nitrogen (Marsh, Fingerhut & Miller, 1965) was measured terminally. The volume and specific gravity of the urine excreted in 2 hr after a water load of 25 ml/kg were measured; they were also measured after water deprivation for 6 and 16 hr.

After 90 days, all rats were killed and autopsies were carried out. Macroscopic abnormalities were noted and the liver, kidneys, brain, spleen, heart, gonads, adrenals, pituitary and thyroid were weighed. Paraffin wax sections of these and of lymph nodes, thymus, urinary bladder, stomach, duodenum, ileum, colon, caecum, rectum, pancreas, uterus and muscle were stained with haematoxylin and eosin for microscopic examination.

## RESULTS

*Acute toxicity in rats*

The LD<sub>50</sub> values, calculated by the method of Litchfield and Wilcoxon (1949), are summarized in Table 1. The oral LD<sub>50</sub> (3.40 and 3.00 g/kg in males and females) is higher than that found by Jenner *et al.* (1964). At high doses, death followed soon after convulsions. At lower doses, salivation, vasodilation and reduced motor activity were observed. Similar effects occurred in animals dosed orally and intraperitoneally. At autopsy, no macroscopic abnormalities were seen.

Table 1. *Acute oral and intraperitoneal toxicity of diacetyl in rats*

Route of administration	Sex	LD <sub>50</sub> (g/kg) with 95% confidence limits
Oral	Male	3.40 (3.18-3.64)
	Female	3.00 (2.42-3.72)
Intraperitoneal	Male	0.40 (0.34-0.48)
	Female	0.64 (0.57-0.72)

Diacetyl was administered as a 20% solution in water, and each dose level was given to groups of five rats of each sex.

*Short-term study in rats*

No differences between test and control animals were seen in the condition or behaviour of the rats at any dosage level. Growth was retarded in rats given 540 mg/kg/day, from wk 2 in males and wk 6 in females. This was more evident in males throughout the period (Table 2). Water consumption was significantly increased in both sexes given diacetyl at 540 mg/kg/day from wk 3 onwards and over the last weeks of the study these animals drank 25-30% more fluid each day than the controls.

The terminal haematological investigations showed that at the highest level of treatment the haemoglobin concentration was significantly reduced, the packed cell volume was lowered and the reticulocyte count was increased in both sexes (Table 3). The total leucocyte count was also increased and this was due predominantly to an increase in neutrophils. Animals administered lower dosage levels showed a slight but not significant anaemia, with no indication of an increased leucocyte count. Effects at 45 days were similar to those at 90 days but were less marked.

There were no significant differences between controls and test animals in the levels of urea and enzymes in the serum in either sex (Table 4). At the highest level of treatment, the urine produced after 16 hr of water deprivation was slightly less concentrated than normal in males but not in females.

Significant changes in organ weight were found in animals receiving 540 mg diacetyl/kg/day (Table 5). In general, males had lower absolute organ weights than controls. Their terminal body weights were 25% less than the controls but decreases in organ weight were smaller (5-15%). Thus, when expressed relative to body weight, the organ weights were increased. The exception to this was the absolute weight of the adrenal gland which was significantly increased. In females, the absolute and relative liver, kidney and adrenal weights were increased. The weight of the pituitary gland was increased only when expressed relative to body weight.

Table 2. *Mean values of body weight, food consumption and water consumption of rats treated with diacetyl in doses of 0-540 mg/kg/day for up to 90 days*

Dose level (mg/kg/day)	Body weight (g) at wk			Food consumption (g/rat/day) at wk			Water consumption (ml/rat/day) at wk		
	0†	4	8	12	0†	4	8	12	12
Males	0	108	263	352	404	14.2	17.8	19.3	18.3
	10	104	259	352	402	13.4	18.9	18.4	18.3
	30	105	261	348	393	14.4	18.9	19.1	18.1
	90	104	258	347	398	14.1	19.5	19.7	18.6
	540	103	231***	286***	318***	13.7	17.0	18.7	17.4
Females	0	95	184	234	260	12.9	15.8	16.3	14.7
	10	94	181	233	257	12.9	15.3	16.2	15.2
	30	94	180	232	256	12.7	16.6	15.9	14.7
	90	95	185	230	262	12.6	16.4	16.5	16.4
	540	93	180	216*	238***	12.3	15.3	16.5	14.7
									28.8
									33.6
									28.8
									29.7
									26.2
									26.6
									26.3
									26.5
									25.5
									31.5
									33.3*
									42.4***
									38.1*
									28.5
									30.3
									25.1
									24.3
									28.5
									28.5
									27.8
									31.7
									35.4***
									29.0*
									29.3

† Day 1 of feeding.

Body-weight values are the means for groups of 15 animals. Values of food and water consumption are the means for three cages of five animals. Although growth and food and water consumption were recorded weekly, only values at monthly intervals are included in the Table.

Values marked with asterisks differ significantly (Student's *t* test) from those of controls: \**P* < 0.05; \*\*\**P* < 0.001.

0349

Table 3. *Haematological findings in rats treated with diacetyl in doses of 0-540 mg/kg/day for 7 and 13 wk*

Table 3. Haematological findings in rats treated with melphalan												
Dose level (mg/kg/day)		Hb (g/100 ml)	MetHb (% of Hb)	PCV (%)	RBC (10 <sup>6</sup> /mm <sup>3</sup> )	Retics (% of RBC)	Total (10 <sup>3</sup> /mm <sup>3</sup> )	Leucocytes				
								Differential (%)				
								N	E	B	L	M
Wk 7												
Male	0	15.6	—	48	8.66	1.6	18.090	13	1	0	84	2
	90	15.3	—	48	8.45	1.3	18.760	11	2	0	85	2
	540	12.8***	—	43***	7.82*	2.7**	25.220**	16	0	0	80	4**
Female	0	15.6	—	45	8.11	1.5	16.690	10	1	0	87	2
	90	15.3	—	46	7.97	1.6	15.070	11	1	0	87	1
	540	12.8***	—	43	7.33**	1.9	21.120	17**	1	0	80**	2
Wk 13												
Male	0	15.8	2.6	46	8.89	1.0	6.770	25	4	0	64	7
	30	15.0	2.3	46	8.58	1.0	8.010	25	3	0	64	8
	60	15.2	2.4	46	8.42	1.0	6.530	21	3	0	68	8
	90	15.0	2.4	46	8.40	1.0	6.380	25	3	0	63	9
	540	11.9***	3.9	39***	8.71	1.8*	8.890	33	1	0	56*	10*
Female	0	14.0	2.6	42	7.70	1.5	5.410	19	3	0	71	7
	30	13.8	3.1	42	7.38	1.8	5.260	22	4	0	66	8
	60	13.3	2.7	41	7.43	1.5	4.790	24	4	0	64	8
	90	13.6	2.5	42	7.72	1.7	5.180	19	3	0	72	6
	540	12.7***	3.4	40	7.52	2.1*	8.750***	33***	2	0	57**	8

Hb = Haemoglobin MetHb = Methaemoglobin PCV = Packed cell volume RBC = Red blood cells  
Retics = Reticulocytes N = Neutrophils E = Eosinophils B = Basophils  
L = Lymphocytes M = Monocytes

Values are the means for groups of eight animals at wk 7 and 15 at wk 13, and those marked with asterisks differ significantly (Student's *t* test) from control values: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

Table 4. *Serum and urine analyses of rats treated with diacetyl at 0-540 mg/kg/day for 45 and 90 days*

Dose level (mg/kg/day)	Serum chemistry			Urine										
	Urea nitrogen (mg/100 ml)	GOT (IU)	GPT (IU)	pH	GOT (IU)	Addis count (10 <sup>3</sup> /hr)	Concentration test					Dilution test (0-2 hr)		
							Specific gravity†			Volume (ml)		Volume (ml)	Specific gravity†	
							Initial	0-6 hr	16-20 hr	0-6 hr	16-20 hr			
Wk 7														
Males 0	—	—	—	7.3	3.8	3.4	1.42	6.53	7.76	4.2	0.8	6.0	0.49	
90	—	—	—	8.2	4.5	6.6	1.80	5.58	7.00	3.0	0.9	5.2	0.64	
540	—	—	—	6.9	5.2	4.3	0.79	6.73	5.35	2.7	1.4	5.7	0.77	
Females 0	—	—	—	7.0	4.3	2.2	1.49	5.48	7.39	2.4	0.4	3.5	0.71	
90	—	—	—	7.5	4.7	1.4	1.84	5.91	7.58	1.4	0.5	2.7	1.23	
540	—	—	—	6.5	4.4	5.1	0.81	5.91	6.23	2.6	0.4	2.8	1.72	
Wk 13														
Males 0	16.9	52.0	9.3	7.9	4.0	3.2	2.39	3.81	7.86	3.7	0.7	9.1	0.32	
10	17.5	54.1	9.5	8.4	3.0	3.2	0.77	4.88	7.21	3.5	0.9	6.3	0.40	
30	17.7	54.6	9.8	8.2	3.0	2.7	1.14	3.89	7.51	3.2	0.6	7.2	0.30	
90	16.7	50.1	9.5	8.9	4.3	3.1	1.51	4.16	7.08	2.8	1.3	6.5	0.36	
540	14.4	45.5	9.8	7.6	6.6	3.7	1.62	4.49	5.61	1.9	1.3	7.3	0.40	
Females 0	18.4	48.9	9.7	—	3.3	2.8	2.15	3.81	7.19	2.3	0.4	5.6	0.45	
10	17.6	44.3	9.7	—	3.5	2.8	1.76	4.08	6.94	1.6	0.6	5.6	0.66	
30	17.1	50.9	9.4	—	4.6	3.1	1.83	4.03	7.20	1.4	0.5	6.0	0.47	
90	17.8	40.7	8.9	—	4.0	3.0	2.01	4.67	7.46	1.7	0.6	5.2	0.51	
540	14.4	44.9	9.2	—	8.0	3.9	1.14	4.08	7.80	2.9	0.4	6.5	0.42	

GOT = Glutamic-oxalacetic transaminase GPT = Glutamic-pyruvic transaminase

† Expressed as (specific gravity × 100) - 100.

Results of serum chemistry are the means for groups of 15 animals and those of urine analysis are the means of six animals at 45 days and 15 animals terminally.

Tests for blood, bile salts and reducing substances were negative in all groups.

TOXICITY OF DIACETYL IN RATS  
J. CHITTET, I. R. GRANT, A. E. G. LANSDOWN, P. GRASSO and J. LANGGILLI

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Table 5. Absolute and relative organ weights of rats treated with diacetyl in doses of 0-540 mg/kg/day for 90 days

Sex and dose level (mg/kg/day)	Brain	Heart	Liver	Spleen	Kidneys	Adrenals†	Gonads	Pituitary†	Thyroid	Terminal body weight (g)
<b>Absolute organ weight (g)</b>										
Male 0	1.76	1.36	9.81	0.71	2.76	61.0	3.52	11.0	19.9	413
30	1.75	1.29	9.95	0.73	2.68	60.7	3.42	10.5	19.1	413
60	1.78	1.31	9.87	0.68	2.70	59.7	3.44	10.9	19.3	402
90	1.77	1.40	10.03	0.74	2.80	64.1	3.50	11.4	21.8	410
540	1.66**	1.07***	8.94**	0.57**	2.38***	72.9***	3.36	10.9	16.3**	303***
Female 0	1.68	0.82	6.23	0.57	1.70	63.2	117	12.5	16.5	265
30	1.69	0.82	6.04	0.54	1.56	63.6	135	12.7	15.2	266
60	1.65	0.83	6.18	0.53	1.56	67.6	133	12.0	16.1	262
90	1.67	0.83	6.09	0.55	1.62	68.2	133	12.8	16.7	261
540	1.55***	0.82	7.72***	0.57	1.82*	78.3***	123	13.1	17.0	240**
<b>Relative organ weight (g/100 g body weight)</b>										
Male 0	0.43	0.33	2.43	0.17	0.68	14.8	0.85	2.66	4.81	
30	0.42	0.31	2.41	0.18	0.64	14.7	0.83	2.54	4.62	
60	0.44	0.33	2.46	0.17	0.68	14.8	0.86	2.71	4.80	
90	0.43	0.34	2.45	0.18	0.68	15.6	0.85	2.78	5.28	
540	0.53***	0.34	2.91***	0.19	0.78***	23.7***	1.09***	3.51***	5.28	
Female 0	0.63	0.31	2.38	0.22	0.64	23.8	43.9	4.70	6.29	
30	0.64	0.31	2.27	0.20	0.58	23.9	50.8	4.78	5.71	
60	0.63	0.30	2.36	0.20	0.60	25.7	50.9	4.59	6.15	
90	0.64	0.32	2.33	0.21	0.62	26.4	50.8	4.88	6.35	
540	0.65	0.34	3.16***	0.24	0.76***	33.1***	51.1	5.47**	6.94	

† Values of absolute and relative weights of these organs and gonads in females are expressed in mg and mg/100 g body weight respectively.

‡ Values are the means of groups of 15 animals and those marked with asterisks differ significantly (Student's *t* test) from the control values: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

At autopsy, sloughing of the squamous epithelium of the stomach was seen in all 15 males and in 14 out of 15 females given the highest level of diacetyl. This lesion was slightly more severe in males than in females. Histologically, ulcers in both the squamous and glandular parts (Fig. 1) of the stomach mucosa were found. In the squamous part, these ulcers were associated with hypertrophy (Fig. 2) or with intercellular oedema, which in one animal was severe enough to form a bullous-like lesion.

A variable degree of infiltration by inflammatory cells, predominantly polymorphonuclear, was seen at the base of the ulcerated areas in the necrotic debris. No histological changes occurred in the stomachs of animals treated with the lower doses. There were no other histological differences between test and control animals in any of the tissues examined.

## DISCUSSION

Jenner *et al.* (1964) reported a lower oral LD<sub>50</sub> (1.58 g/kg) than was found in this study (3.0-3.4 g/kg) although signs of toxicity seen in both tests were similar. Jenner *et al.* (1964) administered diacetyl undiluted whereas a 20% solution of diacetyl in water was used in this trial.

In the short-term study, no effect was seen at dose levels up to and including 90 mg/kg/day. In the 540 mg/kg/day dosage group there was a retardation of body-weight gain and an increase in water consumption, these effects being more pronounced in males. It has been shown that dietary restriction of 25% for 90 days led to a decrease in body-weight gain of approximately 18% in rats (Dr. M. Sharratt, personal communication in 1969). Reductions of a similar order were seen in the present study. With the 25% dietary restriction the weights of most organs, except the liver, were increased relative to body weight. However, the increased relative weights of liver, kidney, pituitary and adrenal in the present test were greater than could be accounted for by the reduction in body weight. Moreover, these organs were also enlarged relative to body weight in females, in which the terminal body weight was decreased by only 9% compared with controls.

The anaemia seen at this dose level can be accounted for by haemorrhage from the stomach ulcers, while the raised polymorphonuclear leucocytosis is probably a consequence of infection of the ulcerated areas.

Conditions of acute stress such as myocardial infarction, severe systemic infection and trauma without loss of consciousness are associated with an increased urinary excretion of glucocorticoids indicative of increased adrenocortical activity (Symington, Currie, Curran & Davidson, 1955; Symington, Currie, O'Donnell, Grant, Oastler & Whyte, 1958). In the rat, increased glucocorticoid production, as indicated by increased blood levels, is associated with an increase in adrenal weight (Hatch, Wiberg, Zawidzka, Cann, Airth & Grice, 1965), and Weber & Singhal (1965) showed that administration of cortisone increased the relative liver weight in rats. Constantinides (1951) demonstrated an immediate enlargement of the kidneys in conditions of stress (cold, forced movement and formalin injections) with little or no change in microscopic appearance of these organs. These observations suggest that the increases in liver, adrenal, and kidney weights can be accounted for by the injury to the stomach epithelium. The known inter-relationships between the adrenal glands and the adenohypophysis may account for the increased weight of the pituitary (Doniach, 1960).

This study has shown that the no-effect level of diacetyl given orally to rats for 90 days is 90 mg/kg/day. The likely maximum intake of diacetyl in man has been calculated to be approximately 10 mg/day (Table 6). In this calculation dietary intakes were based on those

Table 6. Estimate of maximum daily intake of diacetyl by man.

Dietary constituent	Possible concentration of diacetyl (ppm)*	Intake of constituent (g/day)	Flavouring intake (mg/day)
Soft drinks	10	500	5.0
Ice cream	20	2.5	0.05
Sugar confectionery	50	40	2.0
Flour confectionery (cakes and biscuits)	50	48	2.4
Jellies	20	0.3	0.006
Butter and margarine	3	35	0.105
Total...			9.561

\*Highest of maximum use levels recommended by seven of the leading manufacturers of flavouring matters.



FIG. 1. Ulceration of the glandular epithelium of the stomach of a male rat given 540 mg diacetyl/kg/day for 90 days. Haematoxylin and eosin  $\times 40$ .

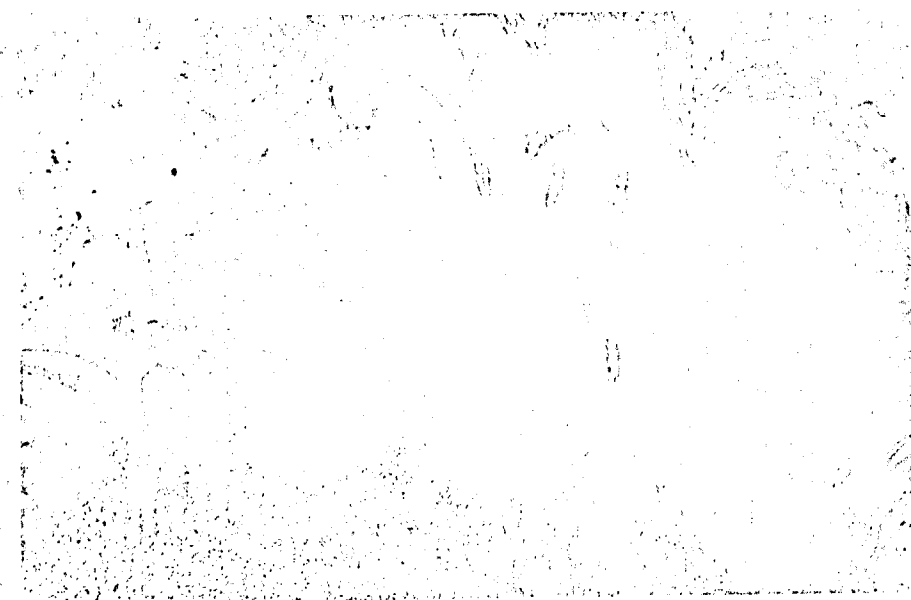


FIG. 2. Hypertrophy of the squamous epithelium of the stomach of a male rat given 540 mg diacetyl/kg/day for 90 days. Haematoxylin and eosin  $\times 40$ .

given by the National Food Survey Committee (1967), except for soft drinks and sugar confectionery which were assumed maxima. The diacetyl concentrations taken are the maxima of those recommended by seven of the leading flavouring manufacturers. The calculated maximum intake level in man (0.17 mg/kg body weight) is approximately 500 times less than the no-effect level (90 mg/kg/day) found in rats in this study.

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### Toxicité aiguë et à court terme du diacétyle chez le rat

**Résumé**—Les  $LD_{50}$  orales et intrapéritonéales aiguës du diacétyle se situent respectivement entre 3,0 et 3,4 g/kg et 0,40 et 0,65 g/kg pour le rat.

Le diacétyle a été administré par intubation orale pendant 90 jours à raison de 0 (animaux témoins), 10, 30, 90 ou 540 mg/kg/jour. Aucun effet défavorable n'a été constaté aux trois dosages inférieurs. Le dosage le plus élevé a provoqué une diminution du gain de poids, une augmentation de la consommation d'eau, de l'anémie, une augmentation de la numération leucocytaire et une augmentation des poids relatifs du foie, des reins et des glandes surrénales et hypophysaires. Les lésions de l'estomac découvertes à l'autopsie montraient une nécrose avec infiltration de cellules inflammatoires.

Le seuil d'indifférence (90 mg/kg/jour) équivaut à environ 500 fois la quantité que l'homme est supposé consommer quotidiennement (0,17 mg/kg).

### Akute und kurzzeitige Toxizität von Diacetyl an Ratten

**Zusammenfassung**—Die akute orale und die intraperitoneale  $LD_{50}$  von Diacetyl an Ratten liegt zwischen 3,0 und 3,4 g/kg bzw. zwischen 0,40 und 0,65 g/kg.

Ratten erhielten 90 Tage lang per Schlundsonde 0 (Kontrolle), 10, 30, 90 oder 540 mg Diacetyl/kg/Tag. Bei den drei niedrigsten Dosierungen wurden keine nachteiligen Wirkungen beobachtet. Die höchste Dosierung verursachte eine Verminderung der Gewichtszunahme, eine Erhöhung des Wasserverbrauchs, Anämie, eine Erhöhung der Leukocytenzahl und eine Zunahme der relativen Gewichte von Leber, Nieren, Nebennieren und Hypophyse. Bei der Autopsie fanden sich am Magen entzündliche, zellig infiltrierte Nekrosen.

Die wirkungsfreie Dosis (90 mg/kg/Tag) entspricht etwa dem 500fachen der geschätzten täglichen Aufnahme durch den Menschen (0,17 mg/kg).

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## Roles of Acetate and Pyruvate in the Metabolism of *Streptococcus diacetilactis*

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*Streptococcus diacetilactis* required acetate, contained acetate kinase and phosphotransacetylase, and incorporated both radioactive exogenous acetate and acetate from citrate into cell lipids. DL- $\alpha$ -Lipoic acid replaced acetate and was required for the oxidation of pyruvate. Stimulation of *S. diacetilactis* by citrate was found to depend on pyruvate oxidation. Resting cells of the organism produced acetate from 73% of the pyruvate they utilized. However, molar growth yields from glucose were not greater under aerobic compared to anaerobic conditions or when lipoic acid or citrate plus lipoic acid was used in the medium in place of acetate. Data indicate that the growth of *S. diacetilactis* is limited by the rate of acetyl-coenzyme A synthesis, that the rate of synthesis from pyruvate is higher than the rate from acetate, and that lack of acetyl-coenzyme A not required for growth limits the production of diacetyl and precludes the formation of adenosine triphosphate from acetyl-coenzyme A.

Collins et al. (5) found that the partially defined medium devised by Niven (27) required addition of acetate or Reticulogen, a refined liver preparation, to support the growth of several strains of *Streptococcus lactis* and *S. cremoris*, organisms closely related to *S. diacetilactis*. Reed et al. (31) found that the growth factor in liver that substituted for acetate in media for *S. lactis* was  $\alpha$ -lipoic acid.

Either acetate or pyruvate can serve as a precursor of acetyl-coenzyme A (CoA). From pyruvate, following decarboxylation and formation of an acetaldehyde-thiamine pyrophosphate (TPP) complex, lipoic acid is involved in transfer of the acyl group of the acetaldehyde-TPP complex to CoA (6, 10, 11, 23, 29). From acetate, there are two mechanisms for the formation of acetyl-CoA. One mechanism occurs in animal tissues, yeast, and some bacteria and involves a single enzyme, acetyl-CoA synthetase (17). The other, restricted to bacteria, involves two enzymes, acetate kinase (19) and phosphotransacetylase (39).

This work was undertaken to define the acetate requirement of *S. diacetilactis* and determine the mechanism of acetate utilization by this organism in relation to pyruvate oxidation and the potential for formation of diacetyl and acetoin by known mechanisms (4, 38).

### MATERIALS AND METHODS

**Organism.** The organism studied was *S. diacetilactis* DRCl (24, 41), which was propagated routinely at

22 C in litmus milk. Identity of the culture was ascertained periodically by checking it for sensitivity to its homologous bacteriophage. *S. faecalis* strain 10 Cl was propagated at 37 C either in litmus milk or on the nutrient agar medium described by Stokstad et al. (40).

**Media.** The partially defined, lipoic acid-free medium used to grow *S. diacetilactis* was the same as that reported by Harvey and Collins (15), except that 0.4% glucose replaced lactose, citrate was omitted, the casein hydrolysate content was increased to 0.2%, pantothenate (1.0  $\mu$ g/ml) and pyridoxal (1.2  $\mu$ g/ml) were included, and the medium was adjusted to pH 6.8. [It has been reported that some casein hydrolysates have low levels of methionine (1), but addition of DL-methionine (1.0  $\mu$ g/ml) to the basal medium did not enhance the growth of *S. diacetilactis*.] In some experiments, DL- $\alpha$ -lipoic acid (1.0  $\mu$ g/ml) replaced sodium acetate (0.2%). The lipoic acid-free basal medium used to grow *S. faecalis* was described by Gunsalus et al. (13). Tryptone broth contained 1.0% tryptone, 1.0% yeast extract, 0.5%  $K_2HPO_4$ , and 0.1% glucose, and was adjusted to pH 6.8.

**Inocula.** Cell suspensions prepared as follows were used as inocula in experiments to determine the acetate-replacing ability of various chemicals and the growth responses of *S. diacetilactis* to acetate or lipoic acid. Cells were harvested from the basal medium (tryptone broth in one experiment) by centrifugation, washed three times in 0.05 M potassium phosphate buffer (pH 6.8), and suspended in basal medium without acetate. Enough cell suspension was added to each flask or test tube of an experiment to give an initial optical density (OD) at 600 nm of 0.01 or 0.02. Growth was considered to be negative in those

flasks or tubes in which the turbidity did not increase during incubation for 36 hr at 32°C.

**Measurement of growth.** Bacterial growth was followed by periodically measuring OD at 600 nm with a Beckman spectrophotometer, model DB, with 1-cm cuvettes. Cell mass was determined from a standard curve relating OD to dry weight. An OD value of 3.72 represented 1 mg (dry weight) per ml for *S. diacetilactis* DRC1 cells harvested in either the exponential or the stationary phase of growth. This relationship was linear up to at least an OD value of 0.6.

Cultures generally were grown statically at 32°C in an atmosphere of air. Aerobic conditions, where indicated, were accomplished by sparging the medium with sterile air passed through a sterile water train at a rate of 20 ml/min. Anaerobic conditions were obtained by purging with sterile nitrogen prior to inoculation and subsequently bubbling nitrogen (99.999% pure) through the medium at a rate of 1 to 2 ml/min.

**Cell-free extracts.** Cells of *S. diacetilactis* were harvested from the indicated medium near the end of exponential growth by centrifugation, washed three times with cold 0.01 M Tris(hydroxymethyl)amino-methane-cysteine (Tris)-cysteine buffer at pH 7.0 (0.001 M with respect to L-cysteine), and resuspended in Tris-cysteine buffer at a cell density of ca. 50 mg (wet weight) per ml. The cells were disrupted with 0.2-mm glass beads (50%, w/w) in a Mickle tissue disintegrator operated at 6- to 8-mm amplitude for 50 min at 4°C. The crude extract was centrifuged at  $15,000 \times g$  for 40 min at 0°C in a refrigerated centrifuge. The supernatant fluid was decanted, dialyzed against 100 volumes of 0.01 M Tris-cysteine buffer (pH 7.0) for 24 hr at 4°C, and stored at 0 or -20°C. For certain indicated experiments, the extracts were treated with anion-exchange resin (acid-washed Dowex 1-X2) to remove CoA (3). [This procedure also removes oxalacetate decarboxylase (15).]

**Analytical procedures.** Acetate kinase and phosphotransacetylase activities in cell-free extracts were determined with the hydroxamic acid method of Seamen (35). One micromole of hydroxamic acid corresponded to an absorbancy of 0.14 at 540 nm. A unit of acetate kinase activity is the amount required to produce 1  $\mu$ mole of hydroxamic acid per min, and specific activity is the number of units per milligram of protein. The reaction exhibited zero-order kinetics for 10 min, and the reaction rate was proportional to concentration of the enzyme up to 0.4 unit per ml. A unit of phosphotransacetylase activity is the amount required to catalyze the arsenolysis of 1  $\mu$ mole of acetyl phosphate per min. Specific activity is number of units per milligram of protein.

Pyruvate oxidation was determined by the method described by Gunsalus et al. (13), which involves measurement of the oxygen consumed by conventional Warburg manometric techniques (43). Oxygen uptake was measured at 37°C for *S. faecalis* and at 32°C for *S. diacetilactis*. The assay systems contained 5 to 6 mg of cells (dry weight). Pyruvic acid was determined by the method of Friedmann and Haugen (9). Acetoin and diacetyl were determined as the sum of both compounds by the method of Westerfeld (45). Citrate was determined by the method of Marier and Boulet

(23). Glucose was determined by the modified anthrone method of Ecker and Lockhart (8). The protein in cell-free extracts was determined by the method of Lowry et al. (20) with bovine serum albumin used as the standard.

Radioactivity measurements were made with an SC-16 windowless flow counter and a Versamatic II scaler (Tracerlab Inc., Waltham, Mass.).

**Chemicals.** Sodium acetate, sodium citrate, sodium arsenite, and potassium arsenate were commercial crystalline reagent grade. The nucleotides, nucleosides, crystalline DL- $\alpha$ -lipoic acid, sodium acetate-2- $^{14}$ C, sodium citrate-1,5- $^{14}$ C, the potassium salt of adenosine triphosphate (ATP), adenosine monophosphate (AMP), adenosine, sodium and potassium pyruvate, and the *N,N'*-dibenzylethylene diamine salt of mevalonic acid were obtained from Calbiochem (Los Angeles, Calif.). CoA and lithium acetyl phosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Free mevalonic acid was prepared from the salt by adjusting an aqueous solution of the salt to pH 11.3 with 5 N NaOH, extracting three times with an equal volume of diethyl ether, and removing the ether in vacuo. Dihydrolipoic acid was prepared from DL- $\alpha$ -lipoic acid with sodium borohydride by the method of Gunsalus et al. (12) as modified by Reiss (32). Vitamin-free casein hydrolysate, crystalline albumin bovine plasma, and *N*-ethylmaleimide were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio).

## RESULTS

**Response to acetate.** *S. diacetilactis* required acetate, though occasionally growth occurred in the partially defined medium lacking acetate. Maximal cell yields (0.298 to 0.306 mg of dry cells per ml) were obtained with acetate concentrations of 0.8 to 2.0 mg/ml, maximal specific growth rates ( $k = 0.658/\text{hr}$ ) occurred with concentrations of 1.0 to 4.0 mg/ml, and the shortest lag time (2 hr) occurred with 2.0 mg/ml. A plot of the maximal yield data indicated that the response of *S. diacetilactis* to acetate was linear, similar to that reported for *Lactobacillus acidophilus* (37). Concentrations of acetate above 8.0 mg/ml generally were inhibitory.

Growth in the absence of acetate occurred more often in tests in which the inoculum was large (0.05 mg of dry cells per ml), but those cultures that grew could not be serially transferred in the medium lacking acetate. Cell yields were low, lag periods were long, and specific growth rates were low ( $k = 0.3/\text{hr}$ ). We were unable to isolate a nonacetate-requiring mutant, and sterilization of the medium by filtration did not prevent the occasional growth in the absence of acetate.

**Substitutes for acetate.** DL- $\alpha$ -lipoic acid, reduced lipoic acid, and natural substances containing lipoic acid (yeast extract and liver ex-

tract) replaced acetate. Maximal cell yields were obtained with DL- $\alpha$ -lipoic acid concentrations of 0.02 ng/ml or greater, and half maximal yields were obtained with 0.01 ng/ml. These concentrations are double those that should have been obtained with L-lipoic acid, since only the L form of lipoic acid is biologically active (16, 44). *S. lactis* and *S. cremoris* required 0.02 ng/ml and 3 to 20 ng/ml, respectively, for half maximal yields (21, 31).

None of the following substances or alterations of the partially defined medium replaced acetate: 10 mg of Tween 80 per ml, 4 mg of sodium citrate per ml, 0.5  $\mu$ g of mevalonic acid per ml (40), 2 mg of  $\beta$ -alanine per ml, 1.2 mg of sodium pyruvate per ml, 0.1 mg of  $\beta$ -lipoic acid per ml (30), omission of ferrous ions (22), increased glucose (42), increased nucleosides and nucleotides (22), or incubation under anaerobic conditions (36).

**Pyruvate oxidation.** We tested *S. diacetilactis* to determine whether this organism has a lipoic acid-dependent pyruvate dehydrogenase system similar to that reported for *S. faecalis* (13, 28, 29). *S. diacetilactis* contains reduced nicotinamide adenine dinucleotide (NADH) oxidase (38), so we used the method of Gunsalus et al. (13). Resting cells of *S. diacetilactis* harvested from the basal medium oxidized pyruvate only when lipoic acid was present (Table 1). Arsenite inhibited the

oxidation, as it did the oxidation of pyruvate by *S. faecalis* (11). Cells of *S. diacetilactis* or *S. faecalis* harvested from tryptone broth, however, oxidized pyruvate without added lipoic acid (Table 1). That these cells had accumulated lipoic acid during growth in the tryptone broth was indicated by the fact that heated extracts prepared from cells of *S. diacetilactis* harvested from tryptone broth replaced lipoic acid in the oxidation of pyruvate by resting cells of *S. faecalis* (Table 1).

Stoichiometry of the reaction mediated by *S. diacetilactis* was indicated by measurements of the pyruvate and oxygen utilized by cells of the organism harvested from the basal medium. Without addition of lipoic acid to the reaction flasks, the resting cells produced acetoin plus diacetyl from 89% of the pyruvate that was utilized. With 3  $\mu$ g of lipoic acid added, they utilized 33.3  $\mu$ moles of pyruvate, consumed 12.1  $\mu$ moles of oxygen, and produced 3.0  $\mu$ moles of acetoin plus diacetyl (from 6.0  $\mu$ moles of pyruvate). The amount of acetate produced, calculated from the amount of oxygen utilized, was 24.2  $\mu$ moles, leaving 3.1  $\mu$ moles of pyruvate unaccounted for in the results.

**Stimulation of growth.** Citrate stimulates the growth rate of *S. diacetilactis* about 35% under some cultural conditions (15). Our results show that stimulation depends on the oxidation of pyruvate (Table 2). *S. diacetilactis* was not stimulated by the addition of citrate (0.1%) to the partially defined lipoic acid-free casein-hydrolysate medium, though an increase in the production of acetoin plus diacetyl showed that the citrate was broken down. With lipoic acid

TABLE 1. Oxidation of pyruvate by resting cells of *S. diacetilactis* or *S. faecalis*

Source of lipoic acid	Amt. (liters) of oxygen consumed per hr per mg of dry cells		
	<i>S. diacetilactis</i> <sup>a</sup>		<i>S. faecalis</i>
	A	B	
None	0	0	0
DL- $\alpha$ -Lipoic acid (10 $\mu$ g)	15	0	135
Residual from tryptone broth <sup>b</sup>	14	0.5	64 <sup>c</sup>
Heated extract of <i>S. diacetilactis</i> cells harvested from basal medium (1.4 mg of protein)			0
Heated extract of <i>S. diacetilactis</i> cells harvested from tryptone broth (2.0 mg of protein)			81

<sup>a</sup> A, no addition; B, sodium arsenite (20  $\mu$ moles) added.

<sup>b</sup> The cells used in these assays were harvested from tryptone broth.

<sup>c</sup> With sodium arsenite (20  $\mu$ moles) added, 1.7 liters of oxygen were consumed per hr per mg of dry cells.

TABLE 2. Influence of lipoic acid on the growth rate of *S. diacetilactis*<sup>a</sup>

Medium alteration <sup>b</sup>	Specific growth rate per hr	Citrate used ( $\mu$ moles/mg, dry wt)	Acetoin plus diacetyl produced ( $\mu$ moles/mg, dry wt)
None	0.682		7.8
Plus citrate (0.1%)	0.546	9.7	20.1
Plus sodium lipoate (10 $\mu$ g/ml), minus sodium acetate	0.768		0.0
Plus sodium lipoate (10 $\mu$ g/ml) and sodium citrate (0.1%); minus sodium acetate	0.785		

<sup>a</sup> Growth temperature was 32°C.

<sup>b</sup> Unaltered medium was the partially defined casein-hydrolysate medium, pH 6.5.

added to the medium in place of acetate, pyruvate produced from glucose could be oxidized, and the organism was stimulated whether or not citrate was added.

**Cell yields.** Resting cells of *S. diacetilactis* converted pyruvate to acetate as did cells of *S. faecalis*, but previous experiments had indicated that *S. diacetilactis* differs from *S. faecalis* by not gaining energy from utilization of citrate or pyruvate (7, 15, 25, 26). Present results substantiated earlier findings. With glucose limited in the basal medium, cell yields per mole were not significantly greater under aerobic compared to anaerobic conditions, with lipoic acid used in place of acetate, or with citrate plus lipoic acid used in place of acetate.

**Activation of acetate.** Dialyzed cell-free extracts of *S. diacetilactis* that had been treated with Dowex 1 to remove CoA activated acetate, and the activation was dependent on ATP. Addition of 12.5 units of CoA to the reaction mixture did not stimulate the activation, indicating that this organism contains acetate kinase rather than acetyl-CoA synthetase. Utilization of acetylphosphate by the extracts depended upon the addition of CoA, showing that they contained phosphotransacetylase. The acetate kinase and phosphotransacetylase activities of extracts prepared from cells grown in tryptone broth were as great as those of extracts prepared from cells grown in the presence of acetate, indicating that these enzymes of *S. diacetilactis* are constitutive.

**Properties of acetate kinase.** The properties of the acetate kinase of *S. diacetilactis* were similar in most respects to those reported for the acetate kinases of other organisms. The optimal pH for its activity in dialyzed Dowex 1-treated cell-free extracts of cultures grown in the partially defined medium, tryptone broth, and tryptone broth plus 0.2% acetate was 7.4, similar to that reported for *Escherichia coli* and *S. hemolyticus* (30) and *Desulfovibrio desulfuricans* (2), somewhat above that reported for *Clostridium acidurici* (34). Treatment of the extracts with ethylenediaminetetraacetate or omission of divalent metal ions from the reaction mixture prevented the activation of acetate.  $Mg^{2+}$  and  $Mn^{2+}$  were equally effective in the assay system,  $Co^{2+}$  was only 35% as effective as  $Mg^{2+}$  or  $Mn^{2+}$ , and  $Ca^{2+}$  was ineffective.

None of the following replaced acetate in the reaction: citrate (640  $\mu$ moles), butyrate (160  $\mu$ moles), propionate (640  $\mu$ moles), or formate (160  $\mu$ moles), similar to the findings for *D. desulfuricans* (2). Neither adenosine diphosphate nor ADP replaced ATP in the forward reaction, substantiating the findings of Rose et al. (33). Methylmaleimide (5  $\mu$ moles) inhibited the en-

zyme, and addition of L-cysteine (10  $\mu$ moles) prior to the addition of N-ethylmaleimide gave protection, indicating, as did the results of Rose et al. (33) and Brown and Akagi (2), that acetate kinase has an essential SH group.

The half-saturation concentrations ( $K_m$ ), determined by conventional Lineweaver-Burk plots (18) in 1.0 M Tris buffer with extracts of cells grown in the basal medium, tryptone broth, and tryptone broth plus 0.2% acetate, were 0.136 M, 0.145 M, and 0.136 M, respectively. These values were close to the value reported for *E. coli* (33) but different from that found for *D. desulfuricans* ( $4.5 \times 10^{-4}$  M; reference 2). Specific activities of the acetate kinase in extracts prepared from cells grown aerobically and anaerobically in a variety of media were essentially the same, indicating that neither the presence of oxygen during growth nor complexity of the growth medium greatly influenced the level of acetate kinase activity.

**Incorporation of acetate into cell material.** *S. diacetilactis* was grown in the following medium supplemented with 1.0  $\mu$ C of acetate-2- $^{14}$ C (37,000 counts/sec) with and without the addition of 0.5% citrate: 0.25% lactose, 0.25% casein hydrolysate, 0.25% yeast extract, 0.50%  $KH_2PO_4$ , 0.10%  $NaHCO_3$ , 0.02%  $MgSO_4$ , and 0.05% acetate, adjusted to pH 6.4. Near the end of exponential growth, the cells from each culture were harvested by centrifugation and washed once with a citrate-lactate-acetoin solution (0.25% of each), once with a pyruvate-bicarbonate solution (0.25% of each), and twice with distilled water. The cells were then suspended in water, and samples were plated for counting. The remaining cells were dried, and the lipids were extracted with hot 95% ethanol by the procedures described by Harvey and Collins (15).

Radioactivity measurements showed that the cells had incorporated acetate into their lipids. Cells that had been grown in the absence of citrate incorporated an amount of radioactive carbon equal to 1.78  $\mu$ moles of acetate per mg of cells (dry weight). Those that had been grown in the presence of citrate incorporated a smaller amount of radioactive carbon, as was expected since acetate and pyruvate are products of citrate (14) and should have diluted the radioactivity. The amount incorporated was equal to 0.57  $\mu$ mole of acetate per mg of cells (dry weight). That acetate from the citrate was in fact incorporated into cell material was established in a similar experiment in which the medium containing 0.5% citrate was supplemented with 1.0  $\mu$ C of citrate-1,5- $^{14}$ C. Radioactivity from the citrate equal to 2.18  $\mu$ moles of acetate per mg of cells (dry weight) was found in the lipid fraction.

## DISCUSSION

The first steps in the synthesis of fatty acids from pyruvate or acetate involve the formation of acetyl-CoA. Results indicate that *S. diacetilactis* forms acetyl-CoA from pyruvate by means of a lipoic acid-dependent dehydrogenase system similar to that reported for *S. faecalis* (13, 28, 29) or from acetate by means of acetate kinase and phosphotransacetylase. With lipoic acid in the partially defined casein-hydrolysate medium in place of acetate, the organism obviously secured sufficient pyruvate for the synthesis of acetyl-CoA, probably from the metabolism of glucose. That pyruvate not required for oxidation of NADH or cell synthesis was derived from the medium containing acetate was indicated by the formation of 7.8  $\mu$ moles of acetoin plus diacetyl (Table 2).

Resting cells of *S. diacetilactis* in the presence of lipoic acid formed acetoin plus diacetyl from only 18% of the pyruvate that was utilized. Most of the remainder (73% of the total), determined from the amount of oxygen utilized in oxidation of the NADH that was formed in the reoxidation of lipoic acid, was converted via acetyl-CoA to acetate. Not more than 9% of the pyruvate that was utilized could have been converted to acetaldehyde since the formation of acetaldehyde from acetyl-CoA would have resulted in a proportional decrease in the consumption of oxygen. This oxidation of pyruvate to acetate probably involved the actions of phosphotransacetylase and the ATP-dependent acetate kinase that we have found the organism to possess. Yet, we found that the cell yield of *S. diacetilactis*, determined under aerobic conditions in the basal medium containing a growth-limiting amount of glucose, was not increased by the addition of lipoic acid plus citrate as a source of pyruvate. These results, which are supported by the results of Harvey and Collins (15), indicate that the growing cells did not get from acetyl-CoA enough ATP to influence molar growth yields. Since the organism has an ATP-dependent acetate kinase, we interpret the results to indicate that in growing cells of *S. diacetilactis* the amount of acetyl-CoA that is available for use in the formation of acetate is severely limited.

The growth rate of *S. diacetilactis* was increased by the substitution of lipoic acid for acetate in the partially defined medium, suggesting that the rate of acetyl-CoA synthesis from pyruvate is higher than the rate from acetate. Nevertheless, the organism incorporated into cell lipids both acetate from citrate-1,5- $^{14}$ C and, also, exogenous acetate-2- $^{14}$ C from a medium that contained citrate. In each of these cases the medium con-

tained 0.25% yeast extract as a source of lipoic acid. This use of acetate as a precursor of acetyl-CoA in the presence of pyruvate and lipoic acid indicates that pyruvate oxidation did not meet entirely the biosynthetic demands of *S. diacetilactis* for acetyl-CoA.

Our results and published molar growth yield data (15, 25) indicate that in growing cells of *S. diacetilactis* very little, if any, of the acetyl-CoA formed from pyruvate is available for the production of ATP. Other data (15) show that excess pyruvate that is not needed for oxidation of NADH or cell synthesis is converted primarily to acetoin plus diacetyl. Together, these results suggest that the formation of acetyl-phosphate from acetyl-CoA is limited in growing cells of this organism and, consequently, that any acetyl-CoA not required for cell synthesis is used primarily for the production of diacetyl rather than acetate. This possibility, however, is not supported by the results showing that resting cells produced acetate from 73% of the pyruvate they utilized.

The production of acetoin does not involve acetyl-CoA, but the production of diacetyl depends on the availability of acetyl-CoA not required for cell synthesis (4, 38). Numerous reports show that *S. diacetilactis* and other organisms that produce diacetyl produce much smaller amounts of it than they do of acetoin, and some microorganisms that produce acetoin do not produce detectable diacetyl (4, 38). Such findings suggest that growing cells of several species besides *S. diacetilactis* produce little acetyl-CoA in excess of that required for cell synthesis.

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# ANTIVIRAL ACTIVITY OF GLYOXALS

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RECENT reports of observed antiviral activity of compounds with an  $\alpha,\beta$ -dicarbonyl structure<sup>1,2</sup> lead us to submit some results of experiments with compounds of this type and of very closely related ones.

In screening compounds for growth-inhibiting activity towards influenza A/WSN/33 (H<sub>1</sub>N<sub>1</sub> strain former designation FM<sub>1</sub>) a number of  $\alpha$ -keto-aldehydes appeared to be active. The experiments were carried out with eleven-day-old embryonated hen's eggs. In each experiment six eggs were used. One hour prior to inoculation of the eggs in the allantoic fluid with 0.1 ml. 10<sup>-5</sup> dilution of a standard seed virus (ID<sub>50</sub> about 7), 0.2 ml. of a 0.1 M solution or suspension of the compound to be tested was injected into the allantoic cavity. The compounds were dissolved or, when necessary, suspended in saline. After incubating for 48 hr. at 36° C. the hemagglutination titre of a pool of allantoic fluids from treated eggs was determined. Dead embryos were discarded. A compound was considered active if the difference between the logarithm of the hemagglutination titre of allantoic fluids from eggs injected with virus and 0.2 ml. saline and the logarithm of the titre of allantoic fluids from treated eggs surpassed -0.6.

From Table I it can be seen that not all glyoxals are active. Compounds with closely related structural features appear to be non-inhibitory in this test. It must be realized that negative results with compounds tested in suspension might have been caused by an insufficient solubility of the compound in the allantoic fluid.

The compounds 3, 4, 17, 20, 21 from Table I appeared to be inactive when tested in the following way. A subtoxic dose was injected into the yolk sac of six-day-old chick embryos. After four hours the eggs were also infected in the yolk sac with the virus. Further incubating at 36° C. showed no difference between the mortality of treated and of control eggs (injected with virus and saline).

Investigation *in vitro* of some active compounds (according to Table I) revealed a direct action on the virus. The virus particles lose their infective power when incubated with glyoxals at low con-

Table I

No.	Compound	Difference between the log hemagglutination titres
1	CH <sub>2</sub> =CO-CHO	2.0 b
2	n-C <sub>4</sub> H <sub>9</sub> -CO-CHO	0.2 c
3	2-CO-CHO	0.23 c
4	p-OH-C <sub>6</sub> H <sub>4</sub> -CO-CHO	2.0 b
5	p-OH-m-NO <sub>2</sub> -C <sub>6</sub> H <sub>3</sub> -CO-CHO	2.3 c
6	p-Br-C <sub>6</sub> H <sub>4</sub> -CO-CHO	0.7 c
7	m-NO <sub>2</sub> -C <sub>6</sub> H <sub>4</sub> -CO-CHO	2.1 c
8	m-NO <sub>2</sub> -C <sub>6</sub> H <sub>4</sub> -CO-CHO	0 b
9	o-Thienyl-CO-CHO	0.1 c
10	o-Naphthyl-CO-CHO	0 c
11	p-OH-C <sub>6</sub> H <sub>4</sub> -CO-C(OH)(H)-H	0.1 b
12	CH <sub>2</sub> =CO-CO-CH <sub>3</sub>	0.4 c
13	i-C <sub>4</sub> H <sub>9</sub> -CO-CO-i-C <sub>4</sub> H <sub>9</sub>	0.0 c
14	p-OH-C <sub>6</sub> H <sub>4</sub> -CO-CO-C <sub>6</sub> H <sub>4</sub> -p-OH	0.1 b
15	p-OH-m-NH <sub>2</sub> -C <sub>6</sub> H <sub>3</sub> -CO-CO-C <sub>6</sub> H <sub>3</sub> -p-OH	0.1 b
16	CH <sub>3</sub> -C(OH)(H)-C(OH)(H)-CH <sub>3</sub>	0.4 b
17	p-OH-C <sub>6</sub> H <sub>4</sub> -CO-C(OH)(H)-CH <sub>3</sub>	0.1 b
18	CH <sub>2</sub> =CO-COOH	-0.1 b
19	2-CO-COOH	0.1 b
20	2-CH <sub>3</sub> -CO-COOH	0 b
21	2-CH <sub>3</sub> -C(OH)(H)-COOH	0.2 b
22	2-CS-COOH	0 b
23	2-C(OH)(H)-COOH	0.1 b
24	p-Br-C <sub>6</sub> H <sub>4</sub> -CH <sub>2</sub> -C(OH)(H)-COOH	0.2 b
25	CH <sub>3</sub> -CO-CO-NH <sub>2</sub>	0.1 c
26	o-Thienyl-CO-CO-C <sub>6</sub> H <sub>4</sub> -H	0.5 c
27	n-C <sub>4</sub> H <sub>9</sub> -CO-CO-C <sub>6</sub> H <sub>4</sub> -H	0 c
28	m-NO <sub>2</sub> -C <sub>6</sub> H <sub>4</sub> -CO-CO-CH <sub>3</sub>	0.3 c
29	m-NO <sub>2</sub> -C <sub>6</sub> H <sub>4</sub> -CO-CO-CH <sub>3</sub> -p-m-NO <sub>2</sub>	-0.3 c
30	Cyclohexane 1,2-dioxime	0.5 c
31	2-Keto-2-phenylacid	0.2 c
32	Isobutyroic acid	-0.1 c
33	Diphenylparabanic acid	-0.4 c
34	Acenochrome	0.2 c

a. In two experiments with a double dose of this compound, which appeared rather toxic, the differences in the logarithms of the hemagglutination titre were 1.6 and 1.0.

b. Tested in solution.

c. Tested in suspension.

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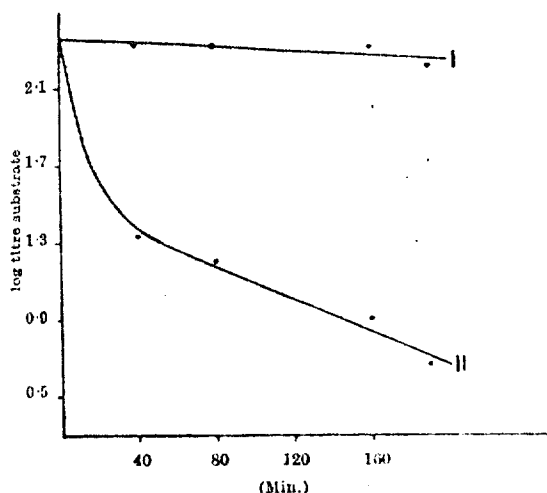


Fig. 1. Action on the substrate urinary mucin of: I, glyoxal-treated FM<sub>1</sub> virus (incubation with 0.001 M *p*-hydroxyphenylglyoxal at 37° C. during 24 hr., followed by dialysis against buffered saline for 3 days at 6° C.); and II, control virus (treated in the same way, but without glyoxal). The hemagglutination titres of I and II were equal. The substrate concentration was determined as the hemagglutination inhibition titre against indicator-2M<sub>1</sub>.

centrations. For example, incubation during 5 hr. at 37° C. in a 0.002 M solution of compound No. 1 (Table I) caused a drop in the ID<sub>50</sub> from 7.5 to negative values. After 1 hr. at 37° C. a 0.004 M solution of compound No. 4 caused a fall of the ID<sub>50</sub> from 8.5 to 6.9.

Somewhat higher concentrations of the glyoxals destroy the enzymic activity of the virus (urinary mucin as a substrate). Removal by dialysis of the excess glyoxal showed that the particles had acquired the properties of indicator virus (Fig. 1).

The hemagglutinating power of the virus is destroyed by still higher concentrations or by incubation during a prolonged time. Incubation of FM<sub>1</sub> virus for 6 hr. at 37° in a 0.004 M solution of compound No. 4 causes no drop of hemagglutination titre. After 24 hr. there was a drop in logarithm of the titre from 2.9 to 2.23; after 54 hr. it was less than 1.25. A control shows no change.

The virucidal action is strong enough to explain the activity in the allantoic test. The negative results in the yolk sac test may be explained in different ways. A more detailed description and discussion of the observed phenomena will be published in the near future.

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## Metabolism of Acetoin in Mammalian Liver Slices and Extracts INTERCONVERSION WITH BUTANE-2,3-DIOL AND BIACETYL

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1. [ $^{14}\text{C}$ ]Acetoin was enzymically synthesized from [ $^{14}\text{C}$ ]pyruvate with a pyruvate decarboxylase preparation. Its optical activity was  $[\alpha]_D^{20} -78^\circ$ . 2. Large amounts (1000-fold higher than physiological concentrations) of acetoin were incubated with rat liver mince. Acetoin disappeared but very little  $^{14}\text{CO}_2$  was evolved. A compound accumulated, which was purified and identified as butane-2,3-diol. Chromatography on borate-impregnated paper indicated the presence of both the *erythro* and *threo* forms. 3. Liver extracts capable of interconverting biacetyl, acetoin and butane-2,3-diol were obtained. These interconversions were catalysed by two different enzymes: acetoin dehydrogenase (EC 1.1.1.5) and butane-2,3-diol dehydrogenase (EC 1.1.1.4), previously identified in bacteria. Both required  $\text{NAD}^+$  or  $\text{NADP}^+$  as cofactors and were different from alcohol dehydrogenase. The equilibrium in both cases favoured the more reduced compound. 4. The activity of butane-2,3-diol dehydrogenase was decreased by dialysis against EDTA; the addition of  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and other bivalent metal ions restored activity. 5. Biacetyl reductase was resolved into multiple forms by CM-Sephadex chromatography and electrophoresis.

Acetoin (3-hydroxybutan-2-one) is produced in significant amounts as a by-product of the mammalian pyruvate decarboxylase reaction (EC 4.1.1.1) (Schweet, Fuld, Cheslock & Paul, 1951). This production is increased by the acetaldehyde formed after the consumption of alcohol (Lubin & Westerfeld, 1945). Acetoin also occurs as a minor constituent of many foodstuffs.

The catabolism of acetoin in mammals has received little attention. Dogs (Greenberg, 1943) and cats (Dawson & Hullin, 1954*a,b*) injected with the compound metabolized it rather rapidly.

Järnefelt (1953, 1955) reported that liver slices, but not homogenates, were capable of converting acetoin into butane-2,3-diol; however, the product was never isolated and was identified solely on the basis of acetaldehyde production in the presence of periodate.

In the present paper we report the enzymic synthesis and purification of [ $^{14}\text{C}$ ]acetoin and the isolation, identification and determination of the isomeric form of the butane-2,3-diol produced on incubation of acetoin with rat liver mince. We also report the preparation of cell-free extracts from mammalian liver capable of reducing acetoin and biacetyl, and the partial purification and some of the properties of the enzymes involved. Our results indicate that in mammalian liver, separate enzymes catalyse the reduction of acetoin and biacetyl.

## MATERIALS AND METHODS

*Preparation of liver mince.* Albino rats of the Sprague-Dawley strain and rabbits were obtained from the animal house of the American University of Beirut. Animals were killed by decapitation and the liver was quickly removed. A mince was prepared by cutting the livers into small pieces (less than 2 mm across) with scissors.

*Preparation of acetone-dried powder extracts.* Rat or rabbit livers (freed from gall bladder) were blended for 1 min with 10 vol. of acetone at  $-18^\circ\text{C}$  and quickly filtered by suction. The cake was reblended twice with the same volume of acetone. Finally it was dried over  $\text{P}_2\text{O}_5$  *in vacuo*; storage was in screw-cap bottles over  $\text{CaSO}_4$  at  $-12^\circ\text{C}$ .

Extracts were prepared by homogenizing the powder for 2 min in a Potter-Elvehjem homogenizer at  $0-4^\circ\text{C}$  with 5 vol. of 0.05 M-sodium phosphate buffer, pH 7.4, and centrifuging at 8000 g for 15 min.

*Fresh liver extract.* Fresh liver was cut into small pieces and homogenized in a Potter-Elvehjem homogenizer at  $0^\circ\text{C}$  with 5 vol. of 0.05 M-sodium phosphate buffer, pH 7.4, and centrifuged at 8000 g for 15 min.

*Chemicals.* [ $^{14}\text{C}$ ]Pyruvate was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Acetoin, obtained from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.), was twice distilled at  $50^\circ\text{C}$  and 2.5 mm Hg. The distillate was left to dimerize at  $4^\circ\text{C}$  and the yellowish-white solid obtained was washed with alcohol and dried *in vacuo*. Butane-2,3-diol obtained

from K & K Laboratories (Plainview, N.Y., U.S.A.) was distilled at 70–80°C and 2–5 mmHg, the first and last fractions being discarded.

Biacetyl (purissima) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.) and used without further purification.

**Radioactivity measurements.** Samples were either dried on concentric planchets and counted for radioactivity in a gas-flow counter with an efficiency of 30% and corrected for self-absorption whenever necessary or added to a sample of a solution of 4g of 2,5-diphenyloxazole and 50mg of 1,4-bis(5-phenyloxazol-2-yl)benzene in 1 litre of toluene and the radioactivity was counted in a liquid-scintillation spectrometer and corrected for efficiency by the internal-standard method. For comparison radioactivity is expressed as d.p.m.

**Determination of acetoin.** Acetoin was determined by the method of Westerfeld (1945). The method was modified for solutions that had been treated with HClO<sub>4</sub> to remove protein, then neutralized with KOH by passing the neutral protein-free filtrate [3 ml of sample + 1 ml of 10% (w/v) sodium tungstate + 1 ml of 0.33 M-H<sub>2</sub>SO<sub>4</sub>] over a mixed-bed [Dowex 1 (Cl<sup>-</sup> form) + Dowex 50 (H<sup>+</sup> form)] column (0.2 cm × 3 cm), washing with water and collecting the first 10 ml of eluate. A portion (5 ml) was taken and subjected to the procedure of Westerfeld (1945).

**Detection of butane-2,3-diol on paper.** When samples were subjected to ascending chromatography on Whatman 3MM paper with ethyl acetate-ethanol-water (12:2:1, by vol.) as the solvent system (Hais & Macek, 1963a) butane-2,3-diol was detected by the periodate-benzidine spray (Hais & Macek, 1963b). However, after chromatography on paper impregnated with borate the dried paper was sprayed lightly with water, suspended for 1 h in a jar containing acetic acid, aerated for 10 min and finally the material detected by the periodate-benzidine method.

**Preparation of [<sup>14</sup>C]acetoin.** [2,3-<sup>14</sup>C]Acetoin was prepared by incubating 15 μmol of [2-<sup>14</sup>C]pyruvate and unlabelled acetaldehyde with 5 ml of pyruvate decarboxylase prepared from ox heart (Green, Westerfeld, Venesland & Knox, 1942) in 0.05 M-glycerophosphate buffer, pH 6.0, containing 0.5 mg of MgSO<sub>4</sub> and 0.1 mg of thiamin pyrophosphate. The enzyme (40–50 mg of protein/ml) was assayed and found to form at least 3 μmol of acetoin/h per ml at saturation with the substrates. Preliminary trials showed that an acetaldehyde/pyruvate molar ratio of 6:1 produced the highest yield of radioactivity in acetoin (up to 60% of the added pyruvate).

A neutralized protein-free filtrate of the incubation mixture was distilled and the distillate passed over a mixed-bed column and distilled again in the presence of dimedone to trap acetaldehyde. The distillate obtained was chromatographed on a column (2 cm × 50 cm) of strongly basic resin [Amberlite IRA-400 (bisulphite form)] prepared as described by Gabrielson & Samuelson (1952) and eluted with water followed by 0.5 M-NaCl (adjusted to pH 7.0).

The pooled eluates in the acetoin peak were concentrated by vacuum distillation in the presence of sodium bisulphite, which complexes the acetoin, followed by the disruption of the acetoin-bisulphite complex with solid Na<sub>2</sub>CO<sub>3</sub> and distillation. The purified aqueous acetoin solution was stored at -12°C. Labelled biacetyl was

prepared by oxidizing a sample of [<sup>14</sup>C]acetoin with the FeCl<sub>3</sub>-FeSO<sub>4</sub>-H<sub>2</sub>SO<sub>4</sub> mixture of Westerfeld (1945). The biacetyl was distilled *in vacuo* and collected at -50°C.

**Identity and radioactive purity of the biosynthesized acetoin.** When [2-<sup>14</sup>C]pyruvate with a specific radioactivity of 31 mCi/mmol was used in the original incubation mixture the purified [2,3-<sup>14</sup>C]acetoin obtained had a specific radioactivity of 27.1 mCi/mmol. The overall radiochemical yield was approx. 20%.

The radioactive purity of the prepared acetoin was checked by its migration as a single peak on Gelite column chromatography in the system of Neish (1950) and on gel-filtration chromatography through a Sephadex G1 (Pharmacia, Uppsala, Sweden) column. Carrier acetoin was added to a sample and the 2,4-dinitrophenyl-oxime and the semicarbazone derivatives were prepared. These were recrystallized to constant specific radioactivity. A sample of the [2,3-<sup>14</sup>C]acetoin was incubated for 2 h with NaIO<sub>4</sub> at pH 4.5 in a tube through which H<sub>2</sub> was passed gently into a trap containing 2,4-dinitrophenylhydrazine. The acetaldehyde (from C-3 and C-4 of acetoin) was collected as the hydrazone in the trap and counted for radioactivity. The acetic acid formed (from C-1 and C-2) was counted for radioactivity in the original solution. The ratio of <sup>14</sup>C in C-2 and C-3 of acetoin was 6:1.

A similar preparation with [3-<sup>14</sup>C]pyruvate gave [1,4-<sup>14</sup>C]acetoin in a similar yield. A large-scale incubation of non-radioactive pyruvate and acetaldehyde was done with the same enzyme preparation and essentially the same purification procedure was used as for the preparation of the radioactive acetoin. The optical rotation of the produced acetoin was [ $\alpha$ ]<sub>D</sub><sup>20</sup> -78°, indicating that it is essentially the D(-) isomer (reported values, [ $\alpha$ ]<sub>D</sub><sup>20</sup> -76° to -79°; Tanko, Munk & Abonyi, 1940). We found this more convenient method of synthesis than the chemical method of Brady, Rabinowitz, Van Baalen & Glick (1951); in addition it yields the D(-)-isomer rather than the racemic mixture.

**Enzyme assays.** All enzyme assays were found to be proportional to enzyme concentration over the range 0.005–0.04 E<sub>340</sub> units/min.

Biacetyl reductase and acetoin reductase activities were assayed by measuring in a recording spectrophotometer (Bausch and Lomb 505) the decrease in E<sub>340</sub> of a mixture of 0.1 ml of 1 M-acetoin or 1 M-biacetyl, 0.1 ml of NADH (1.4 mg/ml) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and 0.7 ml of 0.5 M-sodium phosphate buffer, pH 6.0, in a 1 cm-light-path 1 ml quartz cuvette. The reaction was started by adding 0.1 ml of the enzyme solution to both the reaction cuvette and the blank cuvette. In the blank cuvette the substrate solution was replaced by water. The blank cuvette was placed in the sample holder and the reaction cuvette in the light holder. With higher concentrations of NADH it was necessary to open the slit of the spectrophotometer to admit more light.

The same reactions were assayed in the reverse direction by measuring the increase in E<sub>340</sub> of a mixture containing either 0.1 ml of 1 M-acetoin or 0.1 ml of 30% (v/v) butane-2,3-diol, 0.1 ml of semicarbazide solution (25 mg/ml) (with butane-2,3-diol dehydrogenase), 0.1 ml of Na<sub>2</sub>CO<sub>3</sub> (20 mg/ml) and 0.5 M-sodium pyrophosphate buffer, pH 9.0, to make up to 0.9 ml. The reaction was started

the addition of 0.1 ml of the enzyme solution. The blank contained all the constituents except the substrate.

Alcohol dehydrogenase (EC 1.1.1.1) was also assayed by the same procedure, 0.1 ml of 30% (v/v) propan-1-ol being used as substrate.

**Protein determination.** Protein was determined by the biuret method of Gornall, Bardawill & David (1949) with crystalline bovine albumin as standard. For dilute protein solutions the method of Warburg & Christian (1941) was used.

**Chromatographic separation.** Acetoin, butane-2,3-diol and biacetyl were separated by partition chromatography on Celite columns by using water as the stationary phase and ethyl acetate saturated with water as the mobile phase (Neish, 1950). A portion (45 g) of Celite 545 (Keech-Light Laboratories Ltd.) was triturated with 20 ml of water in a mortar, then packed in a column by adding the powder in small portions and packing with a thick glass rod. The sample was triturated with double its weight of Celite and packed on top and the column was developed with the mobile phase.

## RESULTS

**Accumulation of butane-2,3-diol.** Liver mince (2 g) was incubated at 37°C for 2 h with 22.7  $\mu$ mol of  $[2,3-^{14}\text{C}]$ acetoin ( $1.3 \times 10^6$  d.p.m.) and 4 ml of Krebs-Ringer phosphate buffer (Krebs & Henseleit, 1932) in a flask provided with a well containing sodium hydroxide to trap the  $\text{CO}_2$ . The incubation was terminated by the addition of perchloric acid to a final concentration of 0.3 M. From acetoin determinations in a large number of such incubations it was noted that about 70% of the added acetoin (colour reaction) disappeared. Less than 0.5% of the radioactivity appeared in  $\text{CO}_2$ . These results indicate the accumulation of a metabolite.

**Isolation of butane-2,3-diol.** The protein-free filtrate obtained from the perchloric acid treatment was cooled to 0°C and neutralized with potassium hydroxide. The precipitated potassium perchlorate was centrifuged off and the neutral supernatant passed through a strongly basic resin [Dowex 1 (X8; carbonate form)] column and washed with water. The clear colourless eluate, containing more than 95% of the radioactivity, was passed through a column (1 cm  $\times$  100 cm) of Sephadex G-10 equilibrated and developed with water. Only a single peak could be detected, which moved to the same position as the one obtained with a zero-time control similarly treated. However, when the neutralized Dowex-treated solution was separated by using the same Sephadex G-10 column equilibrated and developed with 0.2 M-sodium bisulphite, two widely separated radioactive peaks were observed (Fig. 1b). The minor peak (first peak) containing about 25% of the radioactivity was identified as the acetoin-bisulphite complex by its colour reaction and the increase in its size when a sample of the protein-free filtrate was mixed with radio-

active acetoin and the separation repeated on the same column (Fig. 1a).

**Identification of butane-2,3-diol.** A preparative-scale reaction mixture was treated and chromatographed as described above for the isolation of butane-2,3-diol. The fractions of the second peak eluted from the Sephadex G-10 column (4 cm  $\times$  100 cm) equilibrated with sodium bisulphite were pooled and authentic butane-2,3-diol was added to the solution. The solution was extracted continuously with peroxide-free ether and the ether was evaporated to give a viscous yellow liquid. The

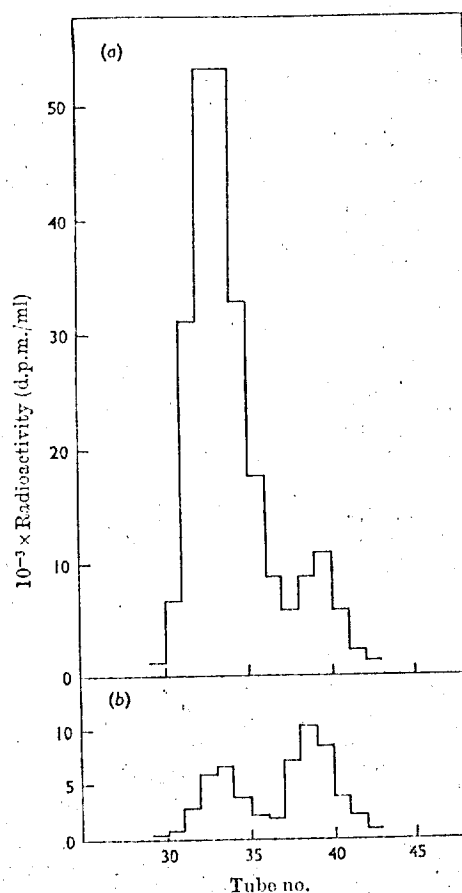


Fig. 1. Chromatography of incubation mixture on Sephadex G-10 equilibrated with bisulphite. The Sephadex G-10 column (1 cm  $\times$  110 cm) was equilibrated and developed with 0.2 M-sodium bisulphite, at a flow rate of 20 ml/h. Fractions (2 ml) were collected. In (b) the 1 ml of incubation mixture was separated; in (a) 1 ml of the same incubation mixture was mixed with 0.1 ml of  $[2,3-^{14}\text{C}]$ acetoin ( $2.7 \times 10^6$  d.p.m.) and separated as described in the text.

liquid was treated with saturated 2,4-dinitrophenylhydrazine in 0.5*M* sulphuric acid, followed by addition of activated charcoal. The preparation was filtered and the clear solution obtained was found to contain more than 90% of the radioactivity of the second peak.

Chromatography of the solution on Whatman 3MM paper with ethyl acetate-ethanol-water (Hais & Macek, 1963*a*) revealed a single coloured spot on development of the chromatogram with the periodate-benzidine method (Hais & Macek, 1963*b*). This spot contained all the radioactivity and also corresponded to the spot obtained with authentic butane-2,3-diol.

A sample of the solution containing 4200 d.p.m. consumed 0.20 mmol of periodate (Birkinshaw, Charles & Clutterbuck, 1931). The solution was gassed with  $N_2$  into a 2,4-dinitrophenylhydrazine trap, and the hydrazone derivative, crystallized three times from hot ethanol, melted at 167°C (m.p. of acetaldehyde hydrazone 167°C). Its specific radioactivity was 35.5 d.p.m./ $\mu$ g, corresponding to the calculated 36 d.p.m./ $\mu$ g if all the radioactivity was in butane-2,3-diol, and was quantitatively cleaved with periodate to 2 mol of acetaldehyde/mol of butane-2,3-diol.

A phenylurethane derivative of the metabolite was prepared and precipitated from hot carbon tetrachloride to a constant specific radioactivity of 11 and 11.2 c.p.m./ $\mu$ g on the second and third precipitations. The derivative did not form uniform crystals.

When the metabolite was chromatographed on borate-impregnated Whatman 3MM paper with ethyl acetate-ethanol-water two radioactive spots with  $R_F$  values of 0.81 and 0.55 were detected. About two-thirds of the radioactivity was distributed in the faster-moving spot and one-third in the slower-moving spot (Fig. 2).

**Properties of extracts.** Liver extracts by themselves did not utilize acetoin. However, the addition of  $NAD^+$ ,  $NADH$ ,  $NADP^+$  or  $NADPH$  to crude cell-free extracts of fresh liver restored activity. These supplemented extracts took up either acetoin or biacetyl (colour reaction of Westerfeld, 1945) at a rate equal to that observed with the equivalent amount of liver mince. The activity with  $NAD^+$  was twice that with  $NADP^+$ . The acetone-dried powder extract was also found to be active on addition of  $NAD^+$  or  $NADP^+$ .

**Partial purification of biacetyl reductase.** Biacetyl reductase was partially purified from rat liver; Table 1 summarizes the procedure. An eightfold purification of biacetyl reductase was obtained with a yield of 35%; some activation occurred at each dialysis step.

The same procedure caused less than twofold purification of acetoin reductase, with a yield of

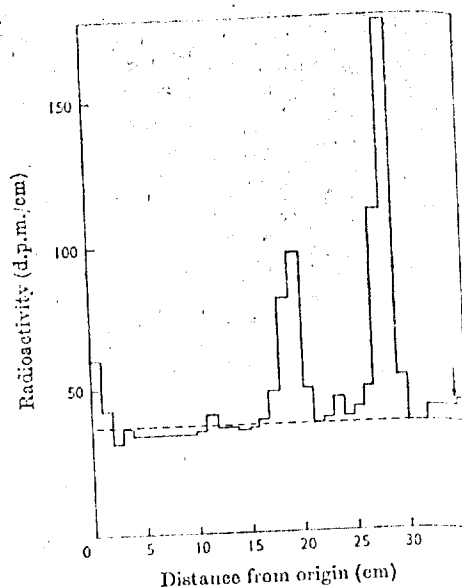


Fig. 2. Ascending chromatography of the metabolite on sodium borate-impregnated Whatman 3MM paper, with ethyl acetate-ethanol-water (12:2:1, by vol.) as solvent. The paper was cut into 1 cm strips and their radioactivity measured in a liquid-scintillation counter. ----, Background counts. The arrow indicates the solvent front.

7%. Similar results were obtained with rabbit liver.

When the purified preparation was applied to a column (5 cm  $\times$  55 cm) of CM Sephadex-50 equilibrated with 0.01 *M* sodium phosphate buffer, pH 6.5, and eluted with the same buffer, the complete profile shown in Fig. 3 was obtained. Biacetyl reductase activity appeared in at least two peaks, none of which was identical with the acetoin reductase peak. Rechromatography of the second large biacetyl reductase fraction on the same column led to a similar separation of the biacetyl reductase into two peaks in about the same positions.

**Effect of ethanol on enzyme activities.** An acetone-dried powder extract of rat liver was divided into three samples. To two samples ethanol was added slowly to a final concentration of 10 and 20% (v/v) respectively, and the solutions were left at 0°C for 10 min and centrifuged at 3000 g for 15 min. The third sample was not treated. All three supernatants were adjusted to 60% saturation with ammonium sulphate; the precipitates were dissolved in sodium phosphate buffer and the activities of alcohol dehydrogenase, butane-2,3-diol dehydrogenase and acetoin dehydrogenase tested. In the untreated crude extract the proportions of the ac-

Table 1. Purification of biacetyl reductase and acetoin reductase from fresh rat liver

The ammonium sulphate fraction was dialysed overnight against 5mM-sodium phosphate buffer, pH 6.5, and applied to a column (2 cm x 25 cm) of DEAE-cellulose equilibrated with 5mM-sodium phosphate buffer, pH 6.5, and eluted with the same buffer. The second dialysate refers to the eluate from the DEAE-cellulose, which was concentrated by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  at 66% saturation and dissolved in 0.01M-sodium phosphate buffer, pH 6.7, and dialysed overnight against the same buffer.

Preparation	Volume (ml)	Protein (mg/ml)	Biacetyl reductase			Acetoin reductase			Biacetyl reductase/acetoin reductase activity ratio
			Specific activity (nmol/min per mg of protein)	Total activity (μmol/min)	Yield (%)	Specific activity (nmol/min per mg of protein)	Total activity (μmol/min)	Yield (%)	
Homogenate	110	55	169	1020	100	3.7	23	100	46
40-60% satd. $(\text{NH}_4)_2\text{SO}_4$	31	41	556	702	68	5.8	7.4	32	95
Dialysate	32	27	887	765	75	5.8	5.0	22	154
After DEAE-cellulose chromatography	60	4	1330	320	31	7.4	1.5	8	180
Dialysate	26	10	1370	340	35	6.4	1.6	7	213

vities were 1:2.7:4.7 respectively. Exposure of the enzyme preparation to 10% (v/v) ethanol destroyed the alcohol dehydrogenase activity completely, but the other two dehydrogenases were not affected significantly.

**Product identification.**  $[^{14}\text{C}]$ biacetyl was incubated with NADH and an acetone-dried powder extract of rat liver for 4h. The product was separated on a Celite column. A double peak representing more than 50% of the radioactivity appeared in the acetoin and butane-2,3-diol regions (Fig. 4). This peak could not be detected in the zero-time control.

The reversal of the acetoin dehydrogenase reaction could not be demonstrated unless an NADH acceptor was included. When  $[^{14}\text{C}]$ acetoin was incubated with NAD $^+$ , phenazine methosulphate, 1mg of Nitro Blue Tetrazolium and an acetone-dried powder extract under the conditions specified in Fig. 5, and the product fractionated on the Celite column, a small peak representing 1.4% of the total radioactivity (c.p.m.) was detected in the biacetyl region. In a control without enzyme the biacetyl region contained only 0.24% of the radioactivity.

**Metal requirement for butane-2,3-diol dehydrogenase.** Dialysis of the enzyme preparation against 1mM-EDTA in 0.01M-sodium phosphate buffer, pH 7.4, followed by dialysis against the phosphate buffer decreased the enzyme activity to one-third of that of a control enzyme preparation dialysed only against the phosphate buffer. The enzyme could be reactivated to 90% of its initial activity by adding  $\text{Co}^{2+}$  to a final concentration of 0.1mM.  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  were slightly less effective at the optimum concentration of 0.1mM, followed by  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ .

**Effect of high NADH concentration.** A sharp decrease in the activity of acetoin reductase occurred when the NADH concentration was increased; at 2mM-NADH the activity was less than 2% of the activity obtained at 0.2mM.

**pH optimum and apparent  $K_m$ .** The pH optimum for both reductase activities was near 6.0. At 0.2mM-NADH and pH 6.0 the apparent  $K_m$  of acetoin reductase was calculated from a Lineweaver-Burk plot to be 50mM for acetoin. The apparent  $K_m$  for biacetyl reductase under the same conditions was 48mM-biacetyl.

**Electrophoresis of acetoin dehydrogenase.** An acetone-dried powder extract of rat liver was subjected to electrophoresis at pH 8.6 on cellulose acetate strips (Millipore Corp., Bedford, Mass., U.S.A.) at 20V/cm for 1h. The position of the biacetyl reductase was located by the application of a second strip moistened with a mixture of 1ml of 1M-acetoin, 0.3ml of phenazine methosulphate (1mg/ml), 1ml of water, 10mg of NAD $^+$  and 3ml of

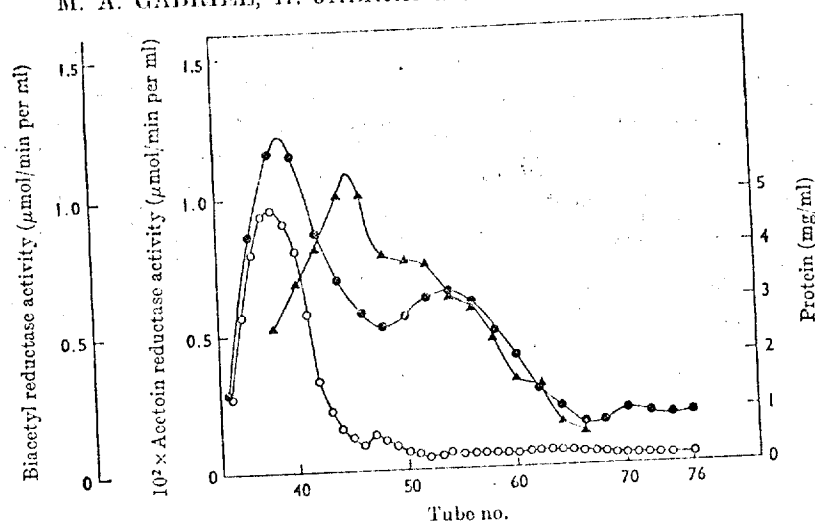


Fig. 3. Separation of acetoin reductase and biacetyl reductase on CM-Sephadex. A portion (10 ml) of the dialysate after DEAE-cellulose treatment (Table 1) was applied to a column (5 cm  $\times$  55 cm) of CM-Sephadex equilibrated for several days and developed with 0.01 M-sodium phosphate buffer, pH 6.7, at a flow rate of 150 ml/h. Fractions (8 ml) were collected. ●, Biacetyl reductase; ○, acetoin reductase; ▲, protein.

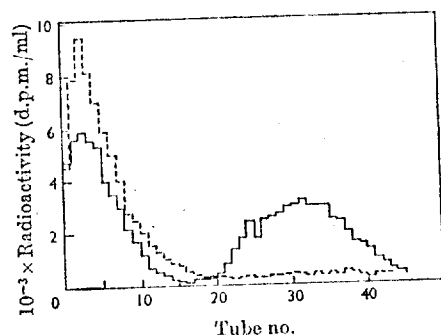


Fig. 4. Celite column chromatography of the incubation mixture of [ $^{14}\text{C}$ ]biacetyl and partially purified biacetyl reductase. [ $1,4\text{-}^{14}\text{C}$ ]Biacetyl (3 nmol; 300 000 d.p.m.) was incubated at 37°C for 4 h with 0.4 mg of NADH, 1.5 ml of 0.1 M-sodium phosphate buffer, pH 7.4, and 0.1 ml of the acetone-dried powder extract. The mixture was separated on a column (2 cm diam.) containing 90 g of Celite 545 and developed with ethyl acetate saturated with water. Fractions (3 ml) were collected. The first peak corresponds to the position of biacetyl and the second to that of acetoin and butane-2,3-diol. —, Experimental; ----, control.

Nitro Blue Tetrazolium (1 mg/ml) (Gelman Instrument Co. Manual no. 701 76-A; P.O. Box 1448, Ann Arbor, Mich., U.S.A.). At least two dark bands of precipitated Nitro Blue and a number of minor bands appeared.

**Stereospecificity of acetoin reductase.** The partially purified acetoin reductase was incubated with NADH and D(-)-acetoin. The butane-2,3-diol formed was chromatographed on borate paper and detected by the modified periodate-benzidine spray (Hais & Maccek, 1963b). Under these conditions acetoin does not give a spot. Two spots with  $R_F$  values of 0.81 and 0.55 were seen.

## DISCUSSION

When liver mince was incubated with labelled acetoin at a concentration of 4.5 mM, which is about 1000-fold the physiological blood concentration of acetoin (Dawson & Hallin, 1954a,b), very little labelled  $\text{CO}_2$  was detected and the major portion of the acetoin (about 70%) was reduced to butane-2,3-diol. At lower concentrations of acetoin much more of it is converted into  $\text{CO}_2$  (M. A. Gabriel & U. A. S. al-Khalidi, unpublished work).

Butane-2,3-diol cannot be separated from acetoin by ordinary gel filtration. However, when the filtration is performed in the presence of bisulphite, which forms a complex with acetoin, the two compounds are well separated. This technique may find general application in the separation of sugars or other compounds when it is possible to form complexes with bisulphite or other complexing agents.

The butane-2,3-diol, produced from D(-)-[ $^{14}\text{C}$ ]acetoin by both liver mince and partially purified



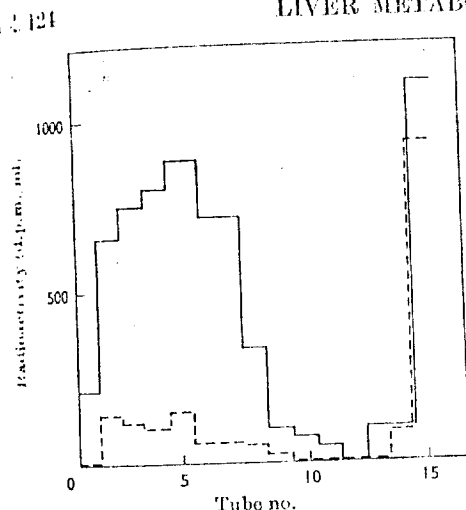


Fig. 5. Celite column chromatography of the incubation mixture of  $[^{14}\text{C}]$ acetoin with acetoin dehydrogenase. Acetoin (13 mmol;  $1 \times 10^6$  d.p.m.) was incubated in a closed tube for 3 hr at  $37^\circ\text{C}$  with 10 mg of  $\text{NAD}^+$ , 0.5 ml of partially purified biacetyl reductase in 0.5 M-sodium pyrophosphate buffer, pH 9.0, 0.5 mg of phenazine methosulphate and 0.2 g of Nitro Blue Tetrazolium in a total volume of 5 ml. The mixture was chromatographed on a column (2.5 cm diam.) containing 68 g of Celite 545 and developed with ethyl acetate saturated with water. Fractions (3 ml) were collected. The first peak coincides with biacetyl. —, Experimental; ----, control.

Acetoin reductase, was separated on borate-impregnated paper into two spots with  $R_F$  values of 0.55 and 0.81. It is assumed that the spot with  $R_F$  0.55 represents the *erythro* form (*meso*) reacting with borate to yield a more polar compound with retarded mobility. The other spot would represent the *threo* (D and/or L) form. Quantitatively the *erythro* spot contained about one-third of the radioactivity. As Taylor & Juni (1969) pointed out in their study of butane-2,3-diol dehydrogenase in bacteria, one enzyme and one form of substrate cannot account for the appearance of two forms of butane-2,3-diol. However, the existence of an isomerase or two reductases may yield the two forms.

This is the first time that mammalian enzyme preparations capable of the interconversion of acetoin, biacetyl and butane-2,3-diol have been reported. Juni & Heym (1957) reported that the bacterial acetoin reductase (EC 1.1.1.4) and biacetyl reductase (EC 1.1.1.5) activities reside in one enzyme, in contrast with the report of Strecker & Hary (1954).

We find that in liver extracts different enzymes catalyse the two reactions. Both enzyme activities are distinct from alcohol dehydrogenase, as the

product after CM-Sephadex treatment contained very little alcohol dehydrogenase activity, and ethanol treatment resulted in the complete loss of activity of alcohol dehydrogenase.

The biacetyl reductase was resolved on CM-Sephadex into at least two peaks. Two bands were also seen on electrophoresis. The multiple forms of biacetyl reductase may be explained on the basis of isoenzymes or of equilibrium of multiple forms of the same enzyme. In favour of the second possibility is the finding of the same complex profile on re-chromatography of one of the fractions on CM-Sephadex.

The equilibrium of both reactions is in favour of the reduced compound. The reaction catalysed by biacetyl reductase is almost irreversible.

The apparent  $K_m$  values obtained for both enzymes are extremely high; the expected physiological concentrations of the substrates are probably two or three orders of magnitude lower than these values. This suggests that the enzymes have very little activity under normal conditions. The possibility that both enzymes are primarily specific for other substrates cannot be ruled out. However, in special cases where acetoin (Dawson & Hullin, 1954a,b), biacetyl or ethanol (Lubin & Westerfeld, 1945) is consumed the tissue substrate contents may become high enough to make the activity of the enzymes significant.

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## Studies of Enzyme Kinetics

II. Inhibition of Pyruvic Carboxylase by Certain Analogs of Pyruvic Acid<sup>1</sup>GLEN R. GALE<sup>2</sup>

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A series of structural analogs of pyruvic acid were tested as possible inhibitors of pyruvic carboxylase. It was found that substitutions on the  $\beta$ -carbon atom yielded the most potent inhibitory compounds. A kinetic study of five such inhibitors indicated a progressive irreversible inhibition, preceded in some cases by an immediate reversible phase. Inhibition was apparently mediated at the substrate-binding site.

## INTRODUCTION

As one of a series of investigations of inhibition of enzymes unique in microorganisms by substrate analogs, pyruvic carboxylase was chosen due to the scarcity of data pertaining to its inhibition. Green *et al.* (3), using a highly purified carboxylase, demonstrated inhibition by silver, copper, or mercury ion, whereas acetaldehyde had only a slight depressing effect and iodobutyrate, lactate and arsenate had no effect. Watt and Werkman (5) showed that the enzyme in *Aerobacter aerogenes* and yeast maceration juice was inhibited by phenylpyruvate, and Redemann and Meikle (4) reported that 2,2-dichloropropionic acid was a competitive inhibitor at low concentrations and an uncompetitive one at higher concentrations. The following is a report of the effect of certain other structural analogs of pyruvate on the yeast carboxylase.

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<sup>2</sup>With the technical assistance of Randall L. Harrington and Ann Marie Welch.

## METHODS

Pyruvic carboxylase was partially purified from Fleischmann's type 20-40 dried yeast (Standard Brands) by the method of Green *et al.* (3). After step three of their method, the precipitate was dissolved in 200 ml. of 0.01 *M* citrate buffer at pH 5.9, 76 g. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added, and the resulting suspension was stored at -10°C. For each experiment an aliquot of this was centrifuged at 10,000  $\times g$  for 10 min. at 5°C, the supernatant solution decanted, and the precipitate dissolved in 0.01 *M* citrate buffer, pH 5.9, so that 1.0 ml. of the resulting enzyme solution resulted in an initial velocity of approximately 17  $\mu$ l. CO<sub>2</sub>/min. from 200  $\mu$ moles pyruvic acid (Na salt, Sigma) in a reaction volume of 2.2 ml. at 30°C. and pH 5.9 with 0.01 *M* citrate buffer. In assays of each batch of enzyme purified, a linear relationship between *v* and enzyme concentration with intercept at the origin was noted, indicating no inhibitors or activators in the preparations which would influence the results during the test period. There was no decrease in activity upon storage at -10°C.

Analogues of pyruvic acid obtained from commercial sources were: acetamide, acetylurea,  $\alpha$ -bromopropionic acid,  $\alpha$ -chloropropionic acid, oxamile acid,  $\alpha$ -nitrophenylpyruvic acid, and 2,3-butanedione (Eastman); ethyl pyruvate and  $\beta$ -chloropyruvic acid (Bios); propionic acid (Fisher); phenylalanine (Calt. Corp. for Biochemical Research); oxamic acid (Matheson, Coleman and

Bell); oxalacetic acid (Krishell); oxalic acid (Mallinckrodt); sodium phenylpyruvate and DL- $\beta$ -phenyllactic acid (Nutritional Biochemicals); *p*-hydroxyphenylpyruvic acid, glyoxylic acid, and ketomalonic acid (HIM); and  $\alpha$ -ketoglutaric acid (Sigma). All compounds were soluble in citrate buffer at the concentrations used, and the pH of each solution was adjusted as necessary.

Measurements of enzyme activity were done with the Warburg respirometer at 30°C. in vessels of approximately 15 ml. capacity. The atmosphere in the vessels was air. Initial velocity,  $v$ , was determined by plotting  $\log(a - x)$  against time in minutes, where  $a$  = initial pyruvate concentration expressed as  $\mu$ l.  $\text{CO}_2$  which would be produced upon complete decarboxylation ( $= \mu\text{moles} \times 22.4$ ), and  $x$  =  $\mu$ l.  $\text{CO}_2$  produced at any given time. From this, the regression coefficient was calculated, which, when multiplied by the factor for conversion to natural logarithms, was  $k$ , the velocity constant of the reaction. The initial velocity was then computed by multiplying  $k$  by the initial substrate concentration,  $a$ ;  $v$  was expressed as  $\mu$ l.  $\text{CO}_2/\text{min}$ . In all cases control vessels with no drug resulted in a straight line for at least 20 min. when  $\log(a - x)$  was plotted against time, indicating first-order kinetics. This was also true in the presence of the proposed inhibitors, with the exception of 2,3-butanedione. With this a slowing of the velocity occurred after 10 min.; therefore, only readings during the first 10 min. were used in calculating  $v$ .

## RESULTS AND DISCUSSION

### INHIBITORY COMPOUNDS

Of the 20 compounds tested, inhibition was obtained usually with those which contained the basic three-carbon fragment of pyruvate with an unaltered  $\alpha$ -ketocarboxylic group, i.e.,  $\beta$ -substituted pyruvate (Table I). These were  $\beta$ -chloropyruvate, phenylpyruvate, *o*-nitrophenylpyruvate, *p*-hydroxyphenylpyruvate, ketomalonate,  $\alpha$ -ketoglutarate and glyoxylate. The only exceptions to this were oxanilic acid, a weak inhibitor, which resembles phenylpyruvate, a nitrogen atom having been substituted for the  $\beta$ -carbon atom, and 2,3-butanedione. The only  $\beta$ -substituted compound tested which failed to show inhibition was oxalacetate.

Substitution of the keto oxygen by two hydrogen atoms (propionate) or one hydro-

TABLE I  
INHIBITION OF THE INITIAL VELOCITY OF PYRUVIC CARBOXYLASE BY SUBSTRATE ANALOGS

Substrate concentration 10.0  $\mu\text{moles}/2.2 \text{ ml.}$  ( $= 4.5 \times 10^{-3} M$ ) throughout. Inhibitors were added to the enzyme 15 min. before addition of substrate.

Analog	Molar concentration	Inhibition
<i>p</i> -Hydroxyphenylpyruvate	$4.5 \times 10^{-3}$	62
<i>p</i> -Hydroxyphenylpyruvate	$1.1 \times 10^{-3}$	71
Chloropyruvate	$4.5 \times 10^{-3}$	27
Chloropyruvate	$2.3 \times 10^{-3}$	73
Glyoxylate	$2.3 \times 10^{-3}$	33
Glyoxylate	$4.5 \times 10^{-3}$	95
<i>o</i> -Nitrophenylpyruvate	$9.0 \times 10^{-4}$	61
<i>o</i> -Nitrophenylpyruvate	$1.1 \times 10^{-3}$	70
Ketomalonate	$2.3 \times 10^{-3}$	71
Ketomalonate	$3.4 \times 10^{-3}$	82
Phenylpyruvate	$1.1 \times 10^{-3}$	68
Phenylpyruvate	$2.3 \times 10^{-3}$	80
Oxanilate	$9.0 \times 10^{-3}$	31
2,3 Butanedione	$9.0 \times 10^{-3}$	56
$\alpha$ -Ketoglutarate	$2.7 \times 10^{-2}$	24

gen and one halogen atom ( $\alpha$ -chloro- or  $\alpha$ -bromopropionate) failed to yield inhibitory compounds. Substitution of this oxygen atom by one hydrogen atom and one amino group, even with a phenyl group on the  $\beta$ -carbon atom (phenylalanine) had no inhibitory effect on the enzyme. Similarly, reduction of phenylpyruvate to phenyllactate resulted in a noninhibitory compound. Substitutions of the carboxyl carbon or the  $\beta$ -carbon atom by amino groups (acetamide and oxamate, respectively), the former by urea (acetylurea), or the latter by a hydroxyl group (oxalate) gave compounds completely without inhibitory activity. Conversion of pyruvate to the ethyl ester yielded an inactive compound.

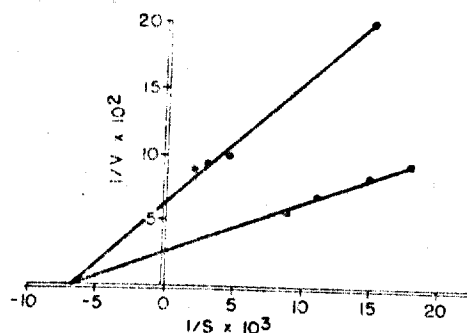


FIG. 1. Reciprocal plot to show typical non-competitive inhibition of pyruvic carboxylase of the type obtained with each of five analogs of pyruvate. Concentration of inhibitor (*o*-nitrophenylpyruvate),  $1.5 \times 10^{-5}$  M. Velocity is  $\mu$ l. CO<sub>2</sub>/min.; substrate concentration is  $\mu$ l. CO<sub>2</sub> which would be evolved upon complete decarboxylation in a final volume of 2.2 ml. The lines are least-squares lines.

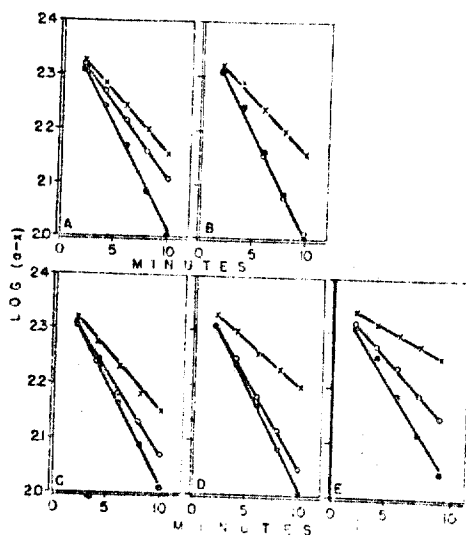


FIG. 2. The course of decarboxylation of pyruvate by pyruvic carboxylase with (●) no inhibitor present, (X) inhibitor added 15 min. before pyruvate, and (○) inhibitor added simultaneously with pyruvate. Inhibitors, with final molar concentrations: A, phenylpyruvate,  $4.5 \times 10^{-5}$ ; B, ketomalonate,  $2.0 \times 10^{-5}$ ; C, *o*-nitrophenylpyruvate,  $4.5 \times 10^{-5}$ ; D, *p*-hydroxyphenylpyruvate,  $4.5 \times 10^{-5}$ ; E, glyoxylate,  $3.2 \times 10^{-5}$ . Pyruvate concentration,  $1.5 \times 10^{-5}$  M. The lines are least-squares lines.

## KINETICS OF INHIBITION

Of the compounds which were inhibitory, five true  $\beta$ -substituted analogs were chosen for a kinetic study of the inhibition. Weak inhibitors (oxamate,  $\alpha$ -ketoglutarate) were omitted, as were  $\beta$ -chloropyruvate due to difficulties encountered in obtaining it in crystalline form, and 2,3-butanedione due to the progressive inhibition with time displayed with this compound. In experiments in which phenylpyruvate, *o*-nitrophenylpyruvate, *p*-hydroxyphenylpyruvate, glyoxylate, or ketomalonate was added to the main compartments of the Warburg vessels and pyruvate tipped in from the side arms 10 min. later after temperature equilibration, it was found that each compound gave results compatible only with noncompetitive inhibition. A typical experiment is plotted in Fig. 1. This indicated either combination of the inhibitor with some site other than the active substrate-binding site, or an irreversible combination at the substrate-binding site. Consequently, experiments were conducted in which each inhibitor was added along with the pyruvate, and 15 min. prior to pyruvate. Results are shown in Fig. 2. In each case simultaneous addition of substrate protected the enzyme from the inhibitor, up to 100% in the case of ketomalonate and *p*-hydroxyphenylpyruvate, indicating that the inhibitor was bound at the substrate-binding site, a situation compatible with the structures of the inhibitors. Furthermore, there appeared to be no inhibition becoming evident in those vessels in which substrate and inhibitor were added simultaneously, as manifest by the strict linearity of the plots of  $\log(a-x)$  against time; that is, the presence of substrate prevented any apparent progressive inhibition. Dixon and Webb (2) state that a reversibly combining substrate can never prevent the ultimate complete inhibition by an irreversible inhibitor, so long as sufficient inhibitor is present to saturate the enzyme, since there is always a small amount of free enzyme as a result of the reversible equilibrium with the substrate. This free enzyme is thus subject to the effects of the inhibitor. The above mentioned linearity of the

plots of  $\log (a - x)$  against time is not necessarily a contradiction of this statement; rather, the period of measurement and experimental error of the method coupled with the relative affinities of the enzyme for each of the compounds likely resulted in inability to detect the progressive inhibition.

Since irreversible inhibition is generally characterized by a progressive increase with time, it was considered of interest to determine the degree of inhibition produced by incubating the enzyme with inhibitor for varying periods of time before addition of substrate. Double side-arm vessels were set up so that each inhibitor could be added to the enzyme at precise times from one side arm before addition of substrate from the other. Figure 3 shows the results expressed as logarithm of velocity (= logarithm of enzyme remaining) after preincubation for specific intervals. A logarithmic plot is used to show the similarity to a first-order reaction. Thermal inactivation of the enzyme was not a factor in these experiments since all vessels were incubated for an equal time at 30°C. regardless of the interval between addition of inhibitor and substrate.

To determine whether any of these five inhibitors was itself decarboxylated, 2.0  $\mu$ moles (45  $\mu$ l.) was added from the side arms of vessels containing three times the usual enzyme concentration. At the end of 2 hr.  $\text{CO}_2$  evolved from each was as follows: glyoxylate, 0; *o*-nitrophenylpyruvate, 0; ketomalonate, 0; *p*-hydroxyphenylpyruvate, 37  $\mu$ l.; phenylpyruvate, 25  $\mu$ l. A plot of  $\log (a - x)$  against time for the two which were decarboxylated showed a decreasing velocity with time.

Inhibition kinetics of the five analogs so studied bear a striking similarity to the kinetics of inhibition of specific acetylcholinesterase by certain organic phosphates (1). Even though these latter compounds do not appear to be structural analogs in the general sense, the mechanism of their inhibition of certain esterases is actually a first-stage hydrolysis, the resulting phosphorylated enzyme being quite inactive (2). Aldridge (1), working with *p*-nitrophenyl diethyl thiophosphate, *p*-nitrophenyl di-

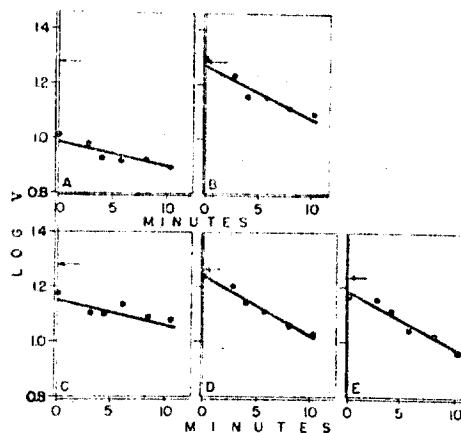


FIG. 3. Progressive decrease in velocity of pyruvic carboxylase by structural analogs of pyruvate when inhibitors were added at certain intervals prior to addition of substrate. Inhibitors, with final molar concentrations: A, phenylpyruvate,  $9.9 \times 10^{-3}$ ; B, ketomalonate,  $9.0 \times 10^{-3}$ ; C, *o*-nitrophenylpyruvate,  $4.5 \times 10^{-3}$ ; D, *p*-hydroxyphenylpyruvate,  $4.5 \times 10^{-3}$ ; E, glyoxylate,  $1.8 \times 10^{-3}$ . Pyruvate concentration,  $4.5 \times 10^{-3}$  M. The arrows indicate velocity in absence of inhibitor. The lines are least-squares lines.

ethyl phosphate, and 8-quinolyl diethyl thiophosphate, found that when either of the first two of these compounds was added to his cholinesterase preparations at certain times before addition of substrate, a plot of logarithm of percentage activity (= logarithm of velocity) against time of incubation yielded a straight line which intercepted the origin. When the quinolyl derivative was used, a straight line again resulted which, however, did not intercept the origin. Such results with this latter compound led him to deduce that the difference between logarithm 100% activity and the actual intercept of the experimental plot was a measure of reversible inhibition: the progressive linear portion of the plot was the irreversible portion. An analogous situation appears evident with carboxylase inhibitors. Phenylpyruvate, *o*-nitrophenylpyruvate, and glyoxylate each yielded a progressive inhibition which did not intercept the origin. The former was the only inhibitor the effects of which were partly reversed by dialysis. Glyoxylate, phenyl-

pyruvate, and its *o*-nitro derivative thus appeared to yield kinetic results similar to the *S*-quinolyl derivative of Aldridge (4) while the other two were more analogous to his *p*-nitrophenyl derivatives.

On the basis of the experimental data presented an exact mechanism of inhibition cannot be inferred. The evidence indicates that the five analogs studied in most detail combine with the active site of the enzyme. The progressive inhibition occurring before addition of substrate could be the result of simply a slow uptake of the inhibitor, the final product being a complex between the enzyme and unaltered analog. Since, however, decarboxylation of two of these apparently occurred, the true inhibitor may have been the aldehyde corresponding to each of the analogs. The fact that decarboxylation of ketomalonate, glyoxylate, or *o*-nitrophenylpyruvate could not be demonstrated does not necessarily invalidate such a proposal since the aldehydes theoretically thus formed were perhaps more potent inhibitors than the others and the total amount of CO<sub>2</sub> evolved before complete inactivation of the enzyme could well have

escaped detection due to limitations of the method. The likelihood that a nondisociable bonding occurs between any such aldehyde formed and the enzyme as with inhibition of cholinesterase by the organic phosphates seems somewhat remote. If this were the case, at any given enzyme concentration the degree of inactivation of the enzyme would be the same upon decarboxylation of a certain number of molecules of any of the inhibitors. The fact that *p*-hydroxyphenylpyruvate was almost completely decarboxylated at the concentration employed, the velocity decreasing with time suggests rather that each molecule of aldehyde, if formed, partially inactivates the active site at which it was formed.

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HISTORICAL REVIEW OF BUTTER "STARTERS"

L. Hart and H. A. Lepper

The term "starters," or "cultures," as used by creamerymen, is understood to refer to cultures of bacteria which induce souring in milk or cream. These cultures contain the same bacteria that produce natural souring, but the process may be controlled by the use of starters. There are, therefore, no new chemical or bacteriological reactions involved, but simply a selection of the proper types of bacteria from those naturally available, so that the reaction proceeds in the desired direction.

The use of starters in the manufacture of butter has arisen naturally. Experience had shown that the inoculation of fresh milk with small quantities of sour milk increased the rate of souring of the fresh milk. A common procedure was to allow a number of samples of milk to sour spontaneously and then select the one with the best flavor and aroma for use in inoculating fresh batches of cream. Another method was to use buttermilk obtained from a previous churning of good butter. These methods were described as early as 1776 as being used in Mongolia. The use of naturally soured cream for butter making is recognized in the first standard for butter issued by the Department in Circular 10, Office of the Secretary. This states: "Butter is the product obtained by gathering in any manner fresh or ripened milk or cream \* \* \*."

By 1850, it had become common practice to use sour cream as a starter. This was usually not specially prepared, and the amounts used for inoculation were more or less a guess. By 1880, bacteriological research began to be applied to dairy processes. Storch, in Denmark, was the pioneer in this work, soon followed, during 1890-1900, by Conn, in this country, and Weigmann, in Germany.

The use of pure cultures of selected bacteria was advocated by Storch. The first commercial starters appeared in 1890, and by 1897, out of the 866 butter plants in that country, 802 used cultures. Originally, pure strains of lactic acid bacteria were advocated.

The isolation of the aroma-producing material of the famous Isigny butter was announced by Biaille in 1894. In 1896, Conn stated that, while the lactic acid in butter is developed from lactose, the flavor, and probably the aroma, comes from other sources. His culture Bacillus No. 41, isolated from milk from Uruguay, was claimed to improve the score of butter:

During 1917-1919, several investigators showed that organisms other than streptococcus lactis are present in starters. Commercial



butter cultures, as now sold, are a mixture of *S. lactis*, the lactic-acid-producing bacteria, with associated organisms. These latter, which convert citric acid to volatile acids, give butter its aroma and flavor. They have been divided into two groups, *S. citrovorus* and *S. paracitrovorus*. These commercial cultures are usually supplied in liquid form. The milk is sterilized, then inoculated, and sent out as requests for cultures are received. Some firms supply commercial cultures in dry form. These are prepared by adding an inert material, such as milk powder, starch or lactose, to a good butter culture, then drying at a low temperature.

A recent development in butter cultures is the isolation of diacetyl and the discovery that this substance is chiefly responsible for the flavor and aroma of butter. Diacetyl is an oxidation product of acetyl methyl carbinol, which is formed by bacterial action from some of the milk constituents, probably citric acid. This is gradually oxidized to diacetyl. In 1929, the Dutch scientists, Van Niel, Kluyver, and Derx, observed that certain bacteria, growing in a special medium, produced a butter-like aroma. Acetyl methyl carbinol was found to be produced in this medium. The oxidation product of this compound, diacetyl, was known to have a butter aroma in very dilute solution. They concluded that it was diacetyl that was chiefly responsible for the aroma of butter. Hammer, at the Iowa Experiment Station, found that satisfactory butter cultures contained comparatively large amounts of diacetyl and of acetyl methyl carbinol, while unsatisfactory cultures contained comparatively small amounts of these constituents. He suggests that either of these compounds be added directly to the butter. He states, however, that there may be some question as to the legality of this practice.

Hammer, in 1933 (Iowa Experiment Station Bulletin 155), developed a special butter culture containing unusually large amounts of acetyl methyl carbinol and diacetyl. This culture was made by inoculating pasteurized milk with a citric acid fermenting streptococcus, allowing it to grow for 15-24 hours, and then adding 0.15 per cent citric acid and 0.3 per cent sulfuric acid. There are now experiments underway to investigate the keeping quality, over extended periods, of butters made with this starter.

The usual procedure in the preparation of butter from ripened cream was to let cream sour at a temperature between 68 and 72° F. until an acidity of 0.6-0.7 per cent as lactic developed. Investigations showed that butter made in this way did not keep as well as sweet cream butter. The method of producing the butter was changed some six years ago to provide for the use of starters, without permitting the acidity of the cream to reach this figure. The culture is first grown in a quart of sterilized milk until coagulation takes place and is then added to 100 gallons of pasteurized skim milk.

The material is allowed to stand until it thickens and develops an acidity of from 0.6-0.7 per cent as lactic. This soured milk is then added to the cream from which the butter is to be made in a proportion of 2-5 per cent, but at a temperature of around 50° rather than at the higher temperature. The cream is kept at this temperature overnight and the acidity develops only to 0.2-0.3 per cent. The churning of this cream into butter is claimed to produce a full flavor without the development of acidity. It is believed that this has much better keeping quality than butter made from cream which is allowed to sour to the higher acidity mentioned above.

Another procedure does not involve letting the cream stand overnight, but rather adding from 2-5 per cent of the cultured skimmed milk to the cooled, pasteurized cream just before it is put into the churn. If the flavor of the cream is quite objectionable, more starter is added to cover this up. Occasionally, the soured milk is added when the fat breaks in the churn and when the batch is trenched to add salt. This method is not often followed as it affects the keeping quality by raising the curd content, and also has the possibility of reducing the fat content if the moisture closely approaches "the limit." This information regarding the practical use of starters was obtained from Mr. Trimble of the Bureau of Dairy Industry, who stated that the procedure of permitting the cream to stand overnight to develop 0.2-0.3 per cent acidity was the one most generally followed.

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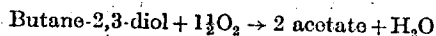
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## The Synthesis of Cell Constituents from Butane-2,3-diol by *Pseudomonas* sp.

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(Received 27 October 1961)

Juni & Heym (1956) showed that the dissimilation of butane-2,3-diol by micro-organisms proceeds via a cyclic mechanism to acetate. Butane-2,3-diol is oxidized to 2-hydroxybutan-3-one and thence to diacetyl, which, in the presence of thiamine pyrophosphate, is hydrolysed to acetate and an 'active acetaldehyde' complex. The complex undergoes an instantaneous condensation with a further molecule of diacetyl to form 3-hydroxy-3-methylpentane-2,4-dione, which is then subsequently reduced to 2,3-dihydroxy-3-methylpentan-4-one; this is hydrolysed to give a second molecule of acetate and butane-2,3-diol. The net effect of one turn of this cycle is to oxidize butane-2,3-diol to acetate according to the following reaction:



Dagley (1958) suggested that the required synthesis of intermediates of the tricarboxylic acid cycle might occur via the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957).

The main purpose of this paper is to show that *Pseudomonas* sp. (tentatively identified as *P. fluorescens*), utilizing butane-2,3-diol as sole source of carbon, incorporates [ $1\text{-}^{14}\text{C}$ ]acetate into cell constituents consistent with the operation of the tricarboxylic acid and glyoxylate cycles. No evidence

has been obtained in support of the direct formation of succinate from acetate (Thunberg, 1920; Glasky, Eicholz & Rafelson, 1958; Glasky & Rafelson, 1957, 1959).

This work has been presented in part to the Biochemical Society (Hullin & Hussall, 1960).

### MATERIALS AND METHODS

**Maintenance and growth of the organism.** The organism was originally isolated from soil, by S. Dagley, on account of its ability to utilize butane-2,3-diol as sole source of carbon for growth. Stock cultures were kept on agar slopes consisting of (w/v):  $\text{KH}_2\text{PO}_4$ , 0.2%;  $(\text{NH}_4)_2\text{SO}_4$ , 0.2%; thiamine hydrochloride, 0.001%;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.04%; butane-2,3-diol, 0.1%; agar, 2.0%; the pH was adjusted to 7.0-7.2 with NaOH. The organism was subcultured every 10-12 weeks, grown at 30°, and stored at 2°; desiccation of the slopes was prevented by sealing the tubes with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2).

Cells were grown for 18 hr. with forced aeration at 30° in 10 l. flasks, containing 9 l. of medium, after inoculation with 800 ml. of a culture grown overnight in the same medium. This medium contained (g./l.):  $\text{KH}_2\text{PO}_4$ , 5.0;  $(\text{NH}_4)_2\text{SO}_4$ , 2.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.4; butane-2,3-diol, 1.8; the pH was adjusted to 7.0-7.2 with NaOH. Cell concentrations were determined turbidimetrically in a Hilger Spekker photoelectric absorptionmeter fitted with Ilford filters neutral II. 50S and blue O.B<sub>2</sub>. The cells were harvested while still in the logarithmic phase of growth (0.25-0.35 mg. dry wt./ml.) using a Sharples continuous-flow centrifuge.

**Manometric studies of oxygen uptake.** The conventional Warburg respirometer was used, as described by Umbreit,

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Barris & Stauffer (1957), to follow the uptake of oxygen during the oxidation of substrates.

*Incorporation of [1-<sup>14</sup>C]acetate into cells utilizing butane-2,3-diol.* The procedure of Kornberg (1958) was closely followed for the purification of the [1-<sup>14</sup>C]acetate and for the subsequent analysis of samples obtained from incubation experiments.

Freshly harvested cells were resuspended (4.3 mg. dry wt./ml.) in medium containing 2.0 mM-butane-2,3-diol, 4.0 mM-phosphate buffer (pH 7.0) and 4.0 mM-NH<sub>4</sub>Cl, and aerated in a water bath at 30°. After 15 min., approx. 60–70% of the diol had been utilized, as ascertained from a sample of the medium incubated at the same temperature in a Warburg respirometer. A portion (0.85 ml.) of the reaction mixture was pipetted into a 80 mm. × 120 mm. centrifuge tube containing 3.0 ml. of ethanol and kept in a water bath at 70°. [1-<sup>14</sup>C]Acetate (50 μC/ml.; 8.3 μC/μmole) (0.96 μmole) was then added and this mixture used as the zero-time sample.

To begin the reaction, 11.5 ml. of the suspension was drawn into a graduated hypodermic syringe (20 ml. capacity), used without the needle, and rapidly transferred to a 50 ml. beaker containing 1.86 ml. of the [1-<sup>14</sup>C]acetate solution. The addition of the cells under pressure provided adequate mixing so that samples could be withdrawn immediately with a second syringe of 1.0 ml. capacity. These samples were quickly ejected into 3.0 ml. of ethanol, the times of addition to the ethanol being recorded from a stop-clock which had been started when the cells were added to the radioactive solution. The reaction vessel was shaken continuously between sampling of the reactants.

*Analysis of samples obtained from incubation experiments.* The aqueous-ethanolic suspensions were analysed by two-dimensional chromatography in 90% (v/v) phenol-formic acid-water (500:13:167, w/v/v) (Kornberg, 1958) and butan-1-ol-propionic acid-water (Calvin & Benson, 1949) as described by Kornberg (1958). The radioactive spots were located by radioautography for 10–30 days with Kodak (Kodirex) X-ray film, and the activity assayed by counting, *in situ*, with a mica end-window β-tube (General Electric Co. type EHM 2/5) standing on a mask with an aperture of up to  $\frac{1}{8}$  in. square. Radioactive compounds were identified by elution and two-dimensional co-chromatography with authentic unlabelled samples of the compound in two solvent pairs. In addition to the two solvents mentioned above the following were also used: butan-1-ol-pyridine-water (1:1:1, by vol.) (Morrison, 1953) and benzene-ether-90% (v/v)-formic acid-water (30:70:14:10, by vol.) (Weimberg, 1959) for carboxylic acids; butan-1-ol-acetone-water-diethylamine (5:5:5:1, by vol.) (Hardy, Holland & Naylor, 1955) for amino acids.

Unlabelled carrier carboxylic acids were located by spraying with a 1.0% (v/v) solution of aniline and 1.0% (w/v) xylose in 95% (v/v) methanol (Nordmann & Nordmann, 1960), and amino acids by spraying with 0.2% ninhydrin in acetone (Smith, 1953) to which pyridine was added to 2.0% (v/v) immediately before use.

*Intensification of radioautographs.* Radioautographs with faint spots, occasionally obtained during co-chromatographic identification, were intensified as described by Moses & Edwards (1960).

*Preparation of cell extracts.* Freshly grown cells were disintegrated in a Hughes (1951) press without abrasive. This crushed material could be stored at –14° for up to a month

without noticeable deterioration or change in characteristics. Each 1.0 g. of crushed cells was ground with 0.5 g. of polishing alumina, grade 3/50 (Griffin and George Ltd., Manchester), and 2.5–3.0 ml. of buffer (KH<sub>2</sub>PO<sub>4</sub>, 2.0 g./l.; adjusted to pH 7.0 with NaOH). This procedure facilitated both the dispersal of the crushed cells and the subsequent clarification of the extract when this was centrifuged at 12 000g for 35 min. at 2°. A pink viscous supernatant solution was obtained with a protein concentration of 30–35 mg./ml., as estimated by the method of Sols (1947), with a solution of crystalline bovine serum albumin for the preparation of a standard curve.

*Determination of diacetyl.* The method used was that of Neuberg & Strauss (1945) as modified by Spencer (1950). It consists in forming the bis-2,4-dinitrophenylhydrazone by heating the sample of diacetyl (0.0–6 μmole in 10 ml.) with 5.0 ml. of 0.02% 2,4-dinitrophenylhydrazine (in 2N-HCl) in stoppered tubes at 100° for 60 min. The resulting precipitate was centrifuged (a process facilitated by the addition of a little kaolin), washed successively with 5N-HCl and water, and the colour developed by twice extracting with 5.0 ml. of 0.3% sodium in ethanol. The pooled extracts were diluted to 25 ml. with sodium ethoxide solution, and the extinction was read at 551 mμ.

*Determination of 3-hydroxy-3-methylpentane-2,4-dione.* This was determined by using acidified ammonium molybdate (Juni & Heym, 1957).

*Determination of glyoxylate and α-oxoglutarate.* These were determined in the presence of each other by the method of Friedemann & Haugen (1943) as modified by Olson (1959).

*Chromatography of 2,4-dinitrophenylhydrazones of keto acids.* 2,4-Dinitrophenylhydrazones of keto acids were chromatographed one-dimensionally in butan-1-ol-ethanol-ammonium carbonate buffer (40:11:14, by vol.) (Dagley, Fewster & Happold, 1952). Further characterization was obtained by spraying the chromatograms with 2.0% (w/v) KOH in aq. 90% (v/v) ethanol.

*Assay of isocitrate lyase (isocitratase) activity.* For each extract a series of dilutions was prepared and each in turn was incubated for 10 min. at 30° in a reaction mixture containing 100 μmoles of DL-isocitrate and 5.0 μmoles of MgSO<sub>4</sub> in a total volume of 1.5 ml. of phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>, 2.0 g./l.; adjusted to pH 7.2 with 5N-NaOH). The reaction was terminated by adding 1.5 ml. of 10% (w/v) trichloroacetic acid, the precipitated protein was removed by centrifuging, and the glyoxylate formed was determined as described above.

*Reactions of [1-<sup>14</sup>C]glyoxylate in cell extracts.* Reactions were carried out in Warburg respirometers filled with nitrogen, and terminated by quickly removing the flasks from the manometers and adding 3.0 ml. of hot (70°) ethanol. The samples, with washings, were centrifuged, and the supernatant solutions evaporated to dryness at 60° under reduced pressure in an atmosphere of nitrogen. Each desiccate was dissolved in 0.2 ml. of aq. 20% (w/v) ethanol brought to 0.1N with HCl, and 0.05 ml. analysed by chromatography with phenol-formic acid-water. The activities of the glyoxylate and malate spots were assayed, as described above, after locating them by radioautography (2–4 days).

*Materials.* Sodium DL-isocitrate was prepared by hydrolysis of the lactone by the method of Olson (1959). Glyoxylate, as the monohydrate of the sodium salt, was synthesized as described by Motzler, Olivard & Snell (1934), and

3-hydroxy-3-methylpentane-2,4-dione (obtained in solution as described by Juni & Heym (1957)). Butane-2,3-diol, 3-hydroxybutane-3-one, diacetyl (all redistilled before use), DL-isocitric acid lactone, ATP, NAD, CoA and acetyl phosphate were obtained from L. Light and Co., Colnbrook, Bucks., and bovine serum albumin from Armour Laboratories, Chicago, U.S.A. Isotopically labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks. Other chemicals used were of AnalaR grade (British Drug Houses Ltd., Poole, Dorset).

## RESULTS

**Dissimilation of butane-2,3-diol to acetate.** Preliminary experiments were designed to confirm that the cycle, proposed by Juni & Heym (1956) for the dissimilation of butane-2,3-diol, operated in *Pseudomonas* sp.

2-Hydroxybutan-3-one and acetate readily replaced butane-2,3-diol as the growth substrate for the organism. No growth was obtained on diacetyl, a result consistent with that of Juni & Heym which, as they suggested, is probably due to the known bacteriostatic effect of diacetyl (Myrvik & Volk, 1954). Suspensions of washed cells, grown on the diol, rapidly oxidized butane-2,3-diol, 2-hydroxybutan-3-one, diacetyl and acetate; cell extracts oxidized the diol and the carbinol at similar rates but diacetyl and acetate only slowly.

The key reaction of the cycle, the synthesis of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl, was shown to occur in cell extracts even under aerobic conditions. The recovery of 3-hydroxy-3-methylpentane-2,4-dione was complete (Table 1), indicating that there was no direct oxidation of diacetyl to acetate. The accumulation of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl in cell extracts is believed to be caused by the inhibition of 3-hydroxy-3-methylpentane-2,4-dione reductase at the concentrations of diacetyl used. The extract (10 ml.) for this experiment was prepared in 0.02M-tris buffer, pH 6.8, and dialysed at 2° against three changes of 2 l. of the same buffer for a total of 6 hr. In this way, inorganic phosphate, which interferes in the estimation of 3-hydroxy-3-methylpentane-2,4-dione and which would be carried through from the growth medium, was removed.

**Incorporation of  $[1-^{14}\text{C}]$ acetate by cells utilizing butane-2,3-diol.** When  $[1-^{14}\text{C}]$ acetate was added to a suspension of whole cells utilizing butane-2,3-diol, isotope was rapidly and linearly incorporated into the cell constituents soluble in aqueous ethanol; a decrease in the rate was observed after the first minute. Incorporation of  $^{14}\text{C}$  into the fraction soluble in aq. ethanol (protein and lipid) was also observed but this occurred at a much lower rate.

**Variation in the percentage distribution of  $^{14}\text{C}$  incorporated from  $[1-^{14}\text{C}]$ acetate into constituents of the**

**soluble fraction.** Analysis of the samples obtained after adding  $[1-^{14}\text{C}]$ acetate to cells utilizing butane-2,3-diol showed that isotope was incorporated into cell constituents in agreement with the concomitant operation of the tricarboxylic acid and glyoxylate cycles (Fig. 1). The initially high and decreasing percentage of the total activity found in citrate and the rapid accumulation of activity in glutamate

Table 1. Formation of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl by extract of *Pseudomonas* sp. grown on butane-2,3-diol

The incubation mixture contained: 105  $\mu$ moles of diacetyl; 10  $\mu$ moles of  $\text{MgSO}_4$ ; 2.0 ml. of dialysed cell extract, 33.6 mg. of protein/ml.; 100  $\mu$ moles of tris, pH 6.8; water to 10 ml. The reaction mixture was incubated aerobically at 30° with shaking in 14 in. x 6 in. boiling tubes, and samples (1.0 ml.) were withdrawn from the mixture at the times given and stopped by the addition of 1.0 ml. of 10% (w/v) trichloroacetic acid.

Time (min.)	Diacetyl utilized ( $\mu$ moles)	3-Hydroxy-3-methylpentane-2,4-dione formed ( $\mu$ moles)	
		Calc.	Found
2	35.6	17.8	18.0
5	67.1	33.5	32.6
10	85.8	42.9	41.1
20	99.0	44.5	43.2
40	105.0	52.5	50.2

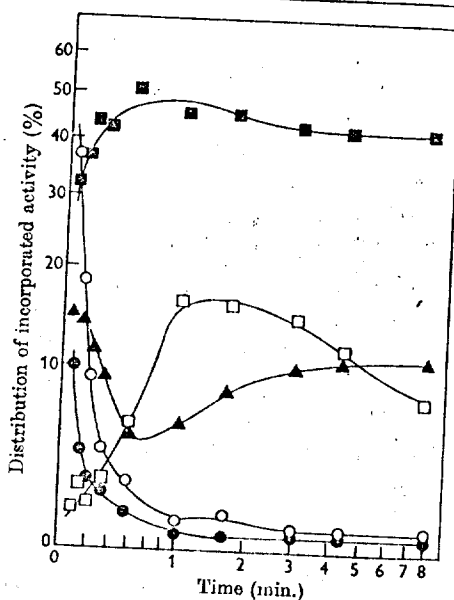


Fig. 1. Variation with time of the percentage distribution of  $^{14}\text{C}$  incorporated from  $[1-^{14}\text{C}]$ acetate into constituents soluble in aq. ethanol of *Pseudomonas* sp. utilizing butane-2,3-diol. O, Citrate; ●, malate; ▲, aspartate; □, succinate; ■, glutamate.

were consistent with the action of the condensing enzyme. Similarly, the decreasing percentage activity of malate and the relatively high initial activity in aspartate suggested the entry of acetate into the tricarboxylic acid cycle via the mediation of malate synthase. The low initial activity of succinate, a compound shown to be present in large amounts by the subsequent high  $^{14}\text{C}$  content, obviated its direct formation from acetate. Labelled phosphates did not appear until 16 sec. after the addition of  $[1-^{14}\text{C}]$ acetate and even after 16 min. did not account for more than 5% of the total incorporated activity.

The appearance of activity in  $\beta$ -hydroxybutyrate was noticeable (1.3%) at  $6\frac{1}{2}$  sec., and was equal to 11.3% of the total at 1 min. and 12.8% at 4 min. 20 sec. The percentage of the total activity present in  $\beta$ -hydroxybutyrate then decreased concurrently with the formation of an unidentified labelled compound with lipid characteristics. This compound ( $R_f$  approx. 0.9–0.95 in both solvents) was probably poly- $\beta$ -hydroxybutyrate, although this was not confirmed.

The only other compound accounting for more than 5% of the total activity at any one time was glutamine. The incorporation of activity into this compound suggested that it was readily in equilibrium with glutamate. The activities of both  $\beta$ -hydroxybutyrate and glutamine have been omitted from Fig. 1 to preserve clarity of presentation.

**Isocitratase activities of cell extracts.** The activity of isocitratase was assayed by estimating the glyoxylate produced from isocitrate under standard reaction conditions. Crude extracts of the organism grown on either butane-2,3-diol or acetate rapidly accumulated both glyoxylate and  $\alpha$ -oxoglutarate, consistent with the action of isocitratase and isocitrate dehydrogenase respectively. Further, after maximum accumulation of glyoxylate, the concentration of this compound decreased with time and finally it disappeared completely from reaction mixtures. This destruction of glyoxylate was later shown to be due, partially, to its reduction to glycinate (H. Hassall & R. P. Hullin, unpublished work). Dialysis of the extract against buffer ( $\text{KH}_2\text{PO}_4$ , 2.0 g./l.; pH 7.2), as described above, reduced both the production of  $\alpha$ -oxoglutarate and the further metabolism of glyoxylate to negligible proportions.

The activity of isocitratase in extracts from cells grown on butane-2,3-diol was identical with that in extracts of cells grown on acetate. This corresponded to a glyoxylate production of  $5.76 \mu\text{moles/hr./mg.}$  of protein and was approximately 20 times that found when succinate was the growth substrate (Fig. 2).

**Synthesis of malate by cell extracts.** The presence of malate synthase was confirmed by demonstrat-

ing the appearance of activity in malate when cell extracts were incubated with  $[1-^{14}\text{C}]$ glyoxylate and various unlabelled additions. Although extracts were first dialysed for a total of 6 hr. as described above in all cases, some  $[^{14}\text{C}]$ glycollate was produced together with traces of other labelled compounds. Nevertheless, certain additions, namely acetate and precursors of acetate with the necessary cofactors, produced a marked effect on the distribution of  $^{14}\text{C}$  between malate and glyoxylate (Table 2).

The necessity of CoA for the synthesis of malate was shown by removing the coenzyme from extracts by treatment with Dowex 2 ( $\text{Cl}^-$  form) (Chantrenne & Lipmann, 1950); the synthesis of malate from glyoxylate, acetate and ATP only occurred if CoA and  $\text{Mg}^{2+}$  ions were added to the incubation mixtures.

Preliminary experiments suggested that malate synthase, in the organism used, was not under adaptive control. Cell extracts of the organism grown on succinate also exhibited malate-synthase activity, indicating that the development of this enzyme, unlike that of isocitratase, was not necessarily dependent on a  $\text{C}_2$  growth substrate, or on a substrate giving rise solely to  $\text{C}_2$  compounds.

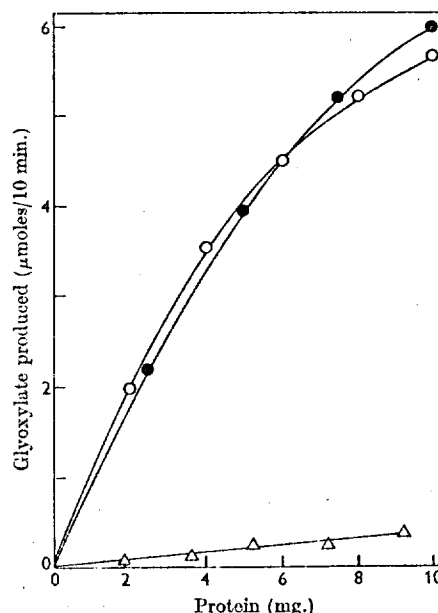


Fig. 2. Isocitratase activities of extracts from cells grown on:  $\bullet$ , butane-2,3-diol;  $\circ$ , acetate;  $\Delta$ , succinate. Each reaction mixture contained  $100 \mu\text{moles}$  of DL-isocitrate,  $5.0 \mu\text{moles}$  of  $\text{MgSO}_4$  and the given amount of extract protein in a total volume of 1.5 ml. of phosphate buffer ( $\text{KH}_2\text{PO}_4$ , 2.0 g./l.; pH 7.2). The mixtures were incubated aerobically at  $30^\circ$  and the reactions terminated after 10 min. by the addition of 1.5 ml. of 10% (w/v) trichloroacetic acid.

Table 2. Incorporation of  $^{14}\text{C}$  from  $[1-^{14}\text{C}]$ glyoxylate into malate by cell extract of *Pseudomonas* sp. grown on butane-2,3-diol

Each incubation mixture contained: 0.4  $\mu\text{mole}$  of sodium  $[1-^{14}\text{C}]$ glyoxylate (0.05  $\mu\text{C}$ ; giving  $5.76 \times 10^3$  counts/min. under the conditions of the radio-assay); 20  $\mu\text{moles}$  of potassium phosphate, pH 7.0; 3.0 mg. of extract protein and additions in a total volume of 1.0 ml.; 0.8  $\mu\text{mole}$  of each addition was used except for NAD (0.2  $\mu\text{mole}$ ). The cell extract had previously been dialysed and preincubated for 30 min. with 2.0 units of CoA and 0.1  $\mu\text{mole}$  of  $\text{MgSO}_4/\text{mg.}$  of protein. The mixtures were incubated at  $30^\circ$  for 45 min. in an atmosphere of nitrogen and the reactions terminated by the addition of 3.0 ml. of ethanol. The activities of the residual glyoxylate and of the malate were determined as described in the Methods section.

Additions	$10^{-3} \times$ Radioactivity (counts/min.)	
	Glyoxylate	Malate
None	5.36	0.22
Acetate	5.34	0.17
ATP	4.98	0.54
Acetate + ATP	0.46	4.18
Diacetyl + ATP	2.24	2.53
2-Hydroxybutan-3-one + ATP	1.82	2.94
Butane-2,3-diol + ATP	2.77	2.09
NAD	5.40	0.20
2-Hydroxybutan-3-one + ATP + NAD	0.26	4.12
Butane-2,3-diol + ATP + NAD	2.40	2.81

### DISCUSSION

The results obtained are consistent with the initial dissimilation of butane-2,3-diol to acetate, followed by the synthesis of all cell constituents from this  $\text{C}_2$  compound; in the latter respect, organisms grown on butane-2,3-diol behave in a manner identical with those grown on acetate. The implications of microbial growth on acetate as sole carbon source, the necessity of net synthesis of  $\text{C}_4$  intermediates of the tricarboxylic acid cycle, and the essential role of the glyoxylate cycle in the metabolism of these organisms, have been discussed fully in recent years (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957; Calley, Dagley & Hodgson, 1958; Collins & Kornberg, 1960; Kornberg, Phizackerley & Sadler, 1960).

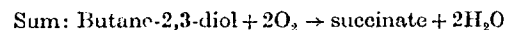
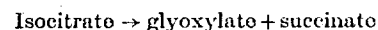
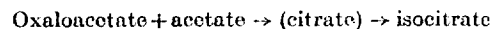
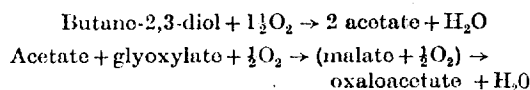
We have shown that whole cells of *Pseudomonas* sp. (probably *P. fluorescens*) growing on butane-2,3-diol rapidly incorporate  $^{14}\text{C}$  from  $[1-^{14}\text{C}]$ acetate consistent with the concomitant operation of the tricarboxylic acid and glyoxylate cycles. The distribution of isotope during the first few seconds of incubation period is in agreement with the entry of acetate into the tricarboxylic acid cycle at two sites, namely, via the mediation of the condensing enzyme to give citrate, and of malate synthase to give malate. The low initial activity of succinate and the correspondingly high initial activity of malate

indicate that little or no malate is formed via succinate. Thus the direct formation of succinate from acetate (Thunberg, 1920; Glasky, Eicholtz & Rafelson, 1958; Glasky & Rafelson, 1957, 1959) plays no obvious part in the synthesis of cell constituents from butane-2,3-diol. The absence of labelled phosphates during the initial incubation periods strongly suggests that carbon dioxide fixation mechanisms are not operative to any large extent during the metabolism of acetate.

The results obtained using cell extracts are also fully consistent with the operation of the glyoxylate cycle. Isocitratase, an enzyme essential for the functioning of the cycle, and shown by many workers (Campbell, Smith & Eagles, 1953; Smith & Gunsalus, 1955, 1957; Olson, 1954, 1959; Saz, 1954; Saz & Hillary, 1956) to be formed only when there is a need of net synthesis of cell constituents from acetate, was present at high levels of activity during growth on butane-2,3-diol (5.76  $\mu\text{moles}$  of glyoxylate formed/hr./mg. of protein). Corresponding activities of the enzyme during growth on acetate and succinate were 5.94 and 0.24 respectively.

The presence in cell extracts of malate synthase, also required for the operation of the glyoxylate cycle (Wong & Aji, 1956), was shown by the incorporation of  $^{14}\text{C}$  from  $[1-^{14}\text{C}]$ glyoxylate into malate. Provided that the necessary cofactors were present, acetate, diacetyl, 2-hydroxybutan-3-one and butane-2,3-diol all increased the amount of  $^{14}\text{C}$  incorporated into malate.

The net synthesis of  $\text{C}_4$  dicarboxylic acids of the tricarboxylic acid cycle can be accomplished from butane-2,3-diol by the following reactions:



The synthesis of  $\beta$ -hydroxy- $[^{14}\text{C}]$ butyrate from  $[1-^{14}\text{C}]$ acetate is assumed to occur via acetyl-CoA and acetoacetyl-CoA. Both of these are fugitive on chromatograms so that the first labelled product appearing would be  $\beta$ -hydroxybutyrate itself. The relatively high accumulation of this compound is of interest owing to its recent implication in terminal respiration (Kulka, Krebs & Eggleston, 1961; Krebs, Eggleston & D'Alessandro, 1961) and its role as a precursor of poly- $\beta$ -hydroxybutyrate, a storage compound in many micro-organisms (Doudoroff & Stanier, 1959; Macrae & Wilkinson, 1958). The polymer can also serve as a substrate for endogenous respiration in certain aerobes (Macrae & Wilkinson, 1958). A radioactive compound with lipid characteristics, formed from  $[1-^{14}\text{C}]$ acetate

during the later stages of the incubation, might possibly have been the polymer, although this was not confirmed.

## SUMMARY

1. The operation of the cyclic pathway whereby butane-2,3-diol is dissimilated to acetate has been confirmed in *Pseudomonas* sp. Whole cells of the organism growing on butane-2,3-diol rapidly oxidize all members of the cycle. Extracts of these cells catalyze the formation of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl.

2. After brief incubation periods, suspensions of the organism utilizing butane-2,3-diol incorporate  $^{14}\text{C}$  from  $[1-^{14}\text{C}]$ acetate only into intermediates of the tricarboxylic acid cycle, directly associated amino acids and  $\beta$ -hydroxybutyrate. The possible polymerization of this compound to give poly- $\beta$ -hydroxybutyrate, recognized as a storage compound in some bacteria, is also considered.

3. The distribution of isotope among the initial products is consistent with the entry of acetate into the tricarboxylic acid cycle at two points, namely via the mediation of the condensing enzyme and of malate synthase. Evidence has also been obtained against the direct formation of succinate from acetate; similarly, the absence of phosphates during the early incubation periods suggests that carbon dioxide-fixation mechanisms play very little part in the synthesis of cell constituents from butane-2,3-diol.

4. Extracts of cells grown on butane-2,3-diol exhibit high activities of isocitratase (5-76  $\mu\text{moles}$  of glyoxylate formed/hr./mg. of protein) equivalent to the activity when acetate is the growth substrate and approximately 20-fold that when succinate is the carbon source. Extracts of the organism grown on butane-2,3-diol rapidly incorporate isotope into malate when incubated with  $[1-^{14}\text{C}]$ glyoxylate, the necessary cofactors, and one of acetate, diacetyl, 2-hydroxybutan-3-one and butane-2,3-diol.

5. These results indicate that the operation of the glyoxylate cycle is essential for the growth of *Pseudomonas* sp. when butane-2,3-diol is the sole source of carbon for growth.

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 METABOLISM OF ACETOIN AND DIACETYL IN LIVER  
 TISSUE<sup>1</sup>

## SOME OBSERVATIONS

by

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(Received for publication September 3, 1953)

Acetoin (acetyl-methylcarbinol) and diacetyl have long been known to be products of the metabolism of living organisms. They have been found in bacteria (1—5), in yeast (20—21) and in animal organisms (6—10). Recently Juni has treated the formation of acetoin in bacteria, yeast and mammalian tissue (11—12). The elimination of acetoin in the animal organism has been treated by Greenberg (15), while Green *et al.* have investigated the elimination of diacetyl in animal tissues (16) and Berl and Bueding the metabolism of acetoin in filariae (17).

In the following preliminary report the writer sets forth some observations on the metabolism of acetoin and diacetyl in liver tissue *in vitro*. It has become apparent that the metabolism of these compounds differs considerably in liver homogenates and in liver slices. The homogenates are incapable of destroying acetoin, while slices have a clear activity towards it. Also diacetyl behaves differently in homogenates and in slices.

## METHODS

Rat livers prepared in two different ways were used throughout this investigation. The homogenates were prepared in a Waring

<sup>1</sup> This investigation has been supported by a generous grant from the Foundation for the Research of Alcohol Problems.

blendor with cold physiological NaCl as the suspending medium and diluted to contain 1 part of liver in 4 parts of homogenate. The liver slices were cut with a razor blade in cold physiological NaCl. In the experiments either 5 ml of the homogenate or 1 g of liver slices in 5 ml of physiological NaCl were used as sources of enzyme. The rest of the reaction mixture was in both cases of the same composition: 3 ml 0.5 phosphate buffer, pH 7.1, and 2 ml (ad 10 ml) of substrates, activators and water. Acetoin and diacetyl were used in 0.1 % solutions (1 ml = 1 mg), Na-pyruvate and acetaldehyde in 1 % solutions (1 ml = 10 mg).  $Mn^{++}$ -ions were added as a 0.5 %  $MnSO_4$  solution. The diphosphothiamine was prepared from thiamine according to Tauber (14). The constituents of the mixture were added in the following order: buffer, activators, pyruvate, liver preparation, substrates, acetaldehyde. Before addition of the substrates the flasks were aerated with tank oxygen to obtain aerobic conditions.

To enable the course of the enzymatic reaction to be followed 1 ml samples were pipetted from the mixture immediately after addition of the substrate and after 30, 60 and 120 minutes had elapsed. Sometimes only the first and last of these samples were taken. For the determination of acetoin and diacetyl in these samples Westerfeld's method (19) in a somewhat modified form was used. The samples were pipetted in 1 ml of 5 % metaphosphoric acid and allowed to stand some time for the precipitation of proteins, then diluted to 10 ml with water and centrifuged. The clear supernatant was distilled according to Westerfeld *in vacuo*, in the presence of NaCl, practically to dryness. A 5 ml aliquot of the distillate was used for the colorimetric determination. Making use of the fact that the colour develops much faster in the case of diacetyl (about 10 min.) than in that of acetoin (60 min.) it was possible to determine acetoin and diacetyl simultaneously by measuring the colour at 10 and 60 min. From measurements with pure acetoin and diacetyl solutions the following formulas were derived:

Take

 $E_{10}$  = the extinction at 10 min. $E_{60}$  = " " " 60 min. $E_x$  = " " produced by acetoin, and $E_y$  = " " " diacetyl.

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Then (the factors were calculated from empirical data)

$$E_x = 1.3 \cdot E_{50} - 1.17 \cdot E_{10}$$

$$E_y = 1.3 \cdot E_{10} - 0.34 \cdot E_{60}$$

From  $E_x$  and  $E_y$  the corresponding amounts of the substances are obtained in the usual manner from an extinction curve. The measurement of the extinctions was performed with a Beckman model B spectrophotometer at a wavelength of 5300 Å. In our experience the method described above is convenient and satisfactory. The accuracy is of course not very high, but seems to be sufficient.

### RESULTS

The results are shown in Tables 1—4. In Table 1 the experiments relating to homogenates are collected. It will be seen that during an incubation time of 2 hours no diminution of acetoin took place, and that acetaldehyde or pyruvate had no effect thereon (exp. 1—4).

TABLE 1  
HOMOGENATES

No.	Added, Theory				DPT 200 γ	MnSO <sub>4</sub> 5 mg	Found, γ			
	Acetal- dehyde mg	Pyruvate mg	Acetoin γ	Diacetyl γ			Acetoin		Diacetyl	
							0 <sup>h</sup>	2 <sup>h</sup>	0 <sup>h</sup>	2 <sup>h</sup>
1	—	—	100	—	—	—	94	97	0.8	0.8
2	1	—	100	—	—	—	110	99	3.6	1.1
3	—	1	100	—	—	—	89	85	1.6	0.8
4	1	—	—	—	—	—	0	7	0	0
5	—	—	—	100	+	+	22	28	62	31
6	1	—	—	100	+	+	28	57	55	32
7	—	1	—	100	+	+	21	36	76	46
8	1	—	—	—	+	+	0	21	0	0

pH 7.4, incubated 2<sup>h</sup> in a water bath at 37 C°.

Diacetyl, however, was clearly active as substrate in homogenates fortified with diphosphothiamine and Mn<sup>++</sup> (exp 5—8). Pyruvate and acetaldehyde seemed to have some effect on the disappearance of diacetyl and the formation of acetoin from it. The ratio between the acetoin formed and the diacetyl which has disappeared varies in the experiments 5—7:

$$5) \frac{+\text{acetoin}}{-\text{diacetyl}} \sim 0.2, \quad 6) \frac{+\text{acetoin}}{-\text{diacetyl}} \sim 0.33, \quad 7) \frac{+\text{acetoin}}{-\text{diacetyl}} \sim 0.5$$

In exp. 6 the acetoin formed by acetaldehyde alone (21γ) is subtracted. Pyruvate alone did not give any increase of acetoin. It was necessary to use homogenates fortified with diphosphothiamine and Mn<sup>++</sup>, as the results in pure homogenates were inconclusive. The high 0<sup>h</sup>-acetoin values in exp. 5—7 are apparently due to impurities in the diacetyl. After distillation of the diacetyl at 88 C° these impurities disappeared as is shown in Table 2, exp. 4—6.

TABLE 2  
SLICES

No.	Added, Theory		Found							
	Acetoin γ	Diacetyl γ	Acetoin, γ				Diacetyl, γ			
			0 <sup>m</sup>	30 <sup>m</sup>	60 <sup>m</sup>	120 <sup>m</sup>	0 <sup>m</sup>	30 <sup>m</sup>	60 <sup>m</sup>	120 <sup>m</sup>
1	100	—	86	78	60	46	1.2	4.8	0	3.6
2	100	—	95	71	70	54	5.2	3.8	0	8.0
3	100	—	86	85	66	52	3.4	4.0	0	5.3
4	—	100	1.4	57	59	59	66	26	13.9	13.1
5	—	100	0.5	59	76	85	77	29	14.7	10.0
6	—	100	5.7	54	66	61	11.4	25	7.2	9.2

pH 7.4, incubated 30—120<sup>m</sup> in a water bath at 37 C.

In Table 2 the corresponding experiments with liver slices are given. One can see that the amount of acetoin has clearly decreased, but that no diacetyl has been formed. (exp. 1—3). In exp. 4—6 a very heavy decrease of diacetyl is seen, which is followed by the formation of an aequimolar amount of acetoin. Contrary to the homogenates the ratio

$$\frac{+\text{acetoin}}{-\text{diacetyl}} \sim 1$$

(The 0<sup>m</sup>-value for diacetyl in exp. 6 is clearly too high, probably due to an experimental error).

In Table 3 the dependence of acetoin disappearance on the initial concentration is shown. It is a curious fact that no disappearance is seen at high initial concentrations (exp. 3—4) while at lower concentrations the phenomenon is clear. Table 4 on the other hand, shows the same experimental series for diacetyl. The reaction

TABLE 3  
SLICES

No.	Acetoin Added, Theory %	Found			
		Acetoin, %		Diacetyl, %	
		0 <sup>h</sup>	2 <sup>h</sup>	0 <sup>h</sup>	2 <sup>h</sup>
1	50	38	34	0.8	2.4
2	200	158	96	8.4	7.4
3	600	155	455	63	21
4	1 000	685	680	40	40

Conditions same as in Table 2.

TABLE 4  
SLICES

No.	Diacetyl Added, Theory %	Found				Acetoin Diacetyl
		Acetoin, %		Diacetyl, %		
		0 <sup>h</sup>	2 <sup>h</sup>	0 <sup>h</sup>	2 <sup>h</sup>	
1	50	0	26	26	2.9	1.1
2	200	0	129	112	13.1	1.3
3	400	0	190	225	56	1.1
4	600	0	306	345	101	1.2

Conditions same as in Table 2.

velocity increases with increasing initial concentration. The ratio between acetoin formed and diacetyl consumed is given in the last column, the values are essentially the same as those derived from Table 2.

#### DISCUSSION

The abovementioned results are not so easy to explain. The lack of activity towards acetoin in the homogenates is probably due to some dilution effect. Another possibility would of course be the destruction of some component of the enzymatic system during the homogenization. As to the mechanism of acetoin disappearance in liver slices it seems fairly clear that acetoin is not oxidized to diacetyl. When acetoin is consumed no increase in diacetyl concentration is observed. Such a situation could be possible, of course, if the diacetyl formed were metabolized with a velocity greater than the velocity of formation. This is actually the case, but during the metabolism of diacetyl an equimolar

(at least) amount of acetoin is invariably formed. This would lead to the paradox that no decrease in acetoin concentration ought to be detected. The mechanism of the metabolism of acetoin is still obscure. In this connexion however, the author wishes to express the following thoughts. A possible line for the metabolism of acetoin could be a splitting of the molecule into two parts, through some mechanism, probably involving coenzyme A, being used for acetylation purposes. The fact that Doisy and Westerfeld have found that acetoin increased the acetylation of p-aminobenzoic acid (18) and that Kinnunen arrived to the same result in the case of sulfonamides (22), can be taken in favour of the hypothesis proposed above.

The fate of diacetyl in metabolism, seems, at least partly, to be a conversion into acetoin. In liver slices this conversion is total. Green *et al.* have described an enzyme, which they call diacetyl mutase (16), and which catalyses the dismutation of 2 moles diacetyl into 1 mole acetoin and 2 moles acetic acid. The quantitative results in our homogenates fit into this system, to some extent at least, but the activity of the slices cannot be explained by it. In this respect the author's experiments could better be explained through some sort of transcarboligation, as described by Akabori and Shimazu (13). In any case it seems clear, that the metabolic pathway of diacetyl primarily goes through acetoin.

Work for a thorough explanation of these problems is in progress.

#### SUMMARY

- 1) A method based on Westerfeld's (19) is given for the simultaneous determination of acetoin and diacetyl in the same sample.
- 2) It is found, that rat liver homogenates do not use acetoin as substrate, while liver slices do this.
- 3) In liver homogenates diacetyl is partially converted into acetoin.
- 4) In liver slices however, all diacetyl which has disappeared, is recovered as acetoin.
- 5) All experiments in this study were carried out under aerobic conditions.
- 6) Some questions, raised by the results above, are discussed.

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## Research Section

### Food Flavourings and Compounds of Related Structure

#### I. Acute Oral Toxicity

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**Abstract**—Oral dosages of 107 synthetic and natural flavourings and structurally-related compounds were administered by intubation to the mouse, rat or guinea-pig. Animals were observed usually for 2 weeks during which time the development of toxic signs was followed and time of death recorded. The acute oral  $LD_{50}$  of each compound was determined.

#### INTRODUCTION

Substances used as food flavourings have received little attention from the toxicological viewpoint. Because of their extensive use as food additives, the Food and Drug Administration has been investigating their toxicity.

The initial step in our toxicity studies was the determination of the acute oral effects. This paper presents data on acute toxicity for a large number of flavouring matters. Similar data are reported for additional compounds, not necessarily flavourings, but included as a means of correlating structure with toxicity. These relationships have been discussed by Taylor, Jenner & Jones (1964) and Hagan, Jenner, Jones & Fitzhugh—*Toxicology*; Long, Brouwer & Webb—*Pathology* (1964).

Flavour additives include compounds with a wide variety of chemical structures, and mixtures of variable composition derived from plants and other natural sources. Some of the substances are synthetic, others are isolates or extracts of natural products. Since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives, a commercially available material was used. No attempt was made to secure chemically pure compounds.

#### METHODS

Groups of 10 young adult Osborne-Mendel rats evenly divided by sex were fasted for approximately 18 hr prior to treatment. Groups of guinea-pigs consisting of both males and females were fasted for the same period. Mice were treated on full stomachs. Animals had access to water at all times, and the food was replaced in cages as soon as animals received their respective doses. All doses were given by intubation.

All animals were maintained under close observation for recording toxic signs and time of death. Such observation was continued until animals appeared normal and showed weight gain. The usual observation period was 2 weeks; in a few cases, where no acute toxic signs were seen, the animals were observed for only one week.  $LD_{50}$ 's were computed by the method of Litchfield & Wilcoxon (1949).

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#### RESULTS

The  $LD_{50}$ 's, slope function, and their confidence limits, together with toxic signs and times of death, are recorded for each compound in Table 1. The species tested, solvent, and solution concentration (w/v) are also listed. Where no solvent is indicated the substance was a liquid and it was administered undiluted.

Table 1. Acute oral toxicity of food flavourings and compounds of related structure

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
ophenone ethyl-1-phenyl ketone)		Rat	3200 (2460-4160)	1.9 (1.3-2.8)	Coma within 5 min persisting in some rats for 24 hr D.T. 1 hr-4 days
hyde C-10 decyl canol)		Rat	>33,320*	—	Excitation, diarrhoea, wet fur on stomach and posterior in both species
		Mouse	>41,750	—	
hyde C-14 undecalactone)		Rat	18,500 (16,930-20,260)	1.2 (1.0-1.3)	Depression within 10 min. Wet fur D.T. 4 hr-5 days
hyde C-18 prunolide		Rat	9780 (7480-12,810)	1.6 (1.4-1.8)	Depression, coma D.T. 4-18 hr
stone of 4-hydroxy-anoic acid)		Guinea-pig	3440 (2890-4100)	1.5 (1.2-1.8)	Depression, salivation D.T. 4 hr-6 days
do.					
acetate†	A†—10%	Rat	142 (116-175)	1.6 (1.3-2.0)	Depression soon after treatment. Rough fur, scrawny appearance for several days D.T. 4 hr-6 days
do.	A—2 & 5%	Mouse	170 (152-190)	1.2 (1.1-1.3)	Depression D.T. 4-18 hr
ctic acid† itenoic acid)	A—15%	Rat	470 (385-573)	1.5 (1.3-1.8)	Depression within 30 min. High dose caused convulsions. Most rats that recover appear normal the day following treatment D.T. 30 min-18 hr
do.	A—15%	Mouse	610 (460-808)	2.2 (1.5-3.1)	Depression D.T. 1-18 hr
lcohol†	B—2%	Rat	70 (63-79)	1.6 (1.2-2.0)	Depression, colourless secretion from eyes, diarrhoea, scrawny appearance for several days D.T. 4 hr-4 days

Highest dose administered

Toxicity was studied because of structural relation to a flavouring agent.

A=corn oil; B=water.

ACUTE ORAL TOXICITY OF FOOD FLAVOURINGS

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Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
4-allylbenzene)		Rat	1820 (1670-1980)	1.2 (1.1-1.3)	Marked depression, some rats in coma for 24 hr, rough fur, wet posterior, porphyrin-like deposit around eyes D.T. 4 hr-8 days
do.	A-10, 20%	Mouse	1250 (812-1920)	1.6 (0.9-2.8)	Depression soon after treatment, coma on higher doses D.T. 1 hr-4 days
benzene†		Rat	5540 (4620-6650)	1.5 (1.2-2.0)	Depression, some rats comatose for 2-3 days, wet posterior, scrawny appearance for several days D.T. 4 hr-5 days
butyrate llyl butanoate)		Rat	250 (216-290)	1.5 (1.2-1.8)	Depression, wet posterior, scrawny appearance for several days D.T. 4 hr-5 days
caproate llyl hexanoate)		Rat	218 (186-255)	1.3 (1.1-1.5)	Depression. Scrawny appearance D.T. 4-18 hr
do.		Guinea-pig	280 (246-319)	1.3 (1.1-1.5)	Depression, salivation D.T. 4 hr-3 days
cinnamate llyl 3-phenylacrylate)		Rat	1520 (1290-790)	1.4 (1.1-1.8)	Scrawny appearance D.T. 4 hr-8 days
cyclohexane opionate llyl 3-cyclohexyl- opionate)		Rat	585 (480-714)	1.5 (1.0-2.2)	Depression, rough fur D.T. 4-hr 6 days
do.		Guinea-pig	380 (172-834)	1.3 (0.5-3.8)	Depression, salivation, haemorrhage in small intestine D.T. 1 hr-6 days
l formate†	A-5%	Rat	124 (107-144)	1.3 (0.5-3.6)	Depression, scrawny appearance for several days D.T. 4 hr-5 days
do.	A-2%	Mouse	136 (122-151)	1.2 (1.1-1.3)	Depression D.T. 4-18 hr
heptylate llyl heptanoate)	A-50%	Rat	500 (392-638)	1.7 (1.2-2.2)	Ataxia D.T. 2-18 hr
do.		Guinea-pig	444 (363-541)	1.6 (1.2-2.3)	Depression D.T. 2-18 hr

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.	A-25%	Mouse	630 (514-772)	1.5 (1.2-1.8)	Depression D.T. 2-18 hr
l isothiocyanate	A-10%	Rat	339 (318-361)	1.6 (1.2-2.1)	Scrawny appearance, porphyrin-like deposit around eyes and nose, rough fur D.T. 4 hr-15 days
l acetate entyl acetate)		Rat	16,550 (14,370-19,030)	1.2 (1.1-1.4)	Depression, coma, rough fur D.T. 4 hr-2 days
l alcohol entanol)		Rat	3030 (1440-6360)	1.2 (1.0-1.4)	Depression D.T. few min-18 hr
yl butyrate entyl butyrate)		Rat	12,210 (10,260-14,560)	1.6 (1.3-1.8)	Depression, rough fur, wet posterior D.T. few min-2 hr
do.		Guinea-pig	11,950 (8530-16,730)	3.0 (1.2-7.2)	Depression, ataxia D.T. 2 hr-6 days
l cinnamic aldehyde -n-pentyl-β-phenyl- rolein)		Rat	3730 (3190-4370)	1.4 (1.2-1.6)	Depression, porphyrin-like deposit around eyes and nose D.T. 4 hr-5 days
yl valerianate entyl pentanoate)		Rat	>35,420*	—	No effect in 2 weeks observation
do.		Guinea-pig	>17,260*	—	Wet fur D.T. 2-6 days
ris oil		Rat	5580 (4540-6860)	1.5 (1.4-1.6)	Ataxia, coma within 1 hr, porphyrin-like deposit around eyes and nose. Wet posterior D.T. 1-3 days
-methoxy-4-propenyl- benzene)		Rat	2090 (1420-3070)	1.8 (1.3-2.4)	Low doses—depression, high doses—coma D.T. 4 hr-4 days
do.		Guinea-pig	2160 (1920-2450)	1.3 (1.2-1.5)	Depression D.T. 1-7 days
do.		Mouse	3050 (2330-4000)	1.6 (1.2-2.1)	Depression, coma within 15 min D.T. 2-4 hr
aldehyde -methoxybenzaldehyde)		Rat	1510 (1360-1700)	1.2 (1.1-1.3)	Depression D.T. 4-18 hr
		Guinea-pig	1260	1.6	Depression within 2 hr

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
benzene)		Rat	3700 (3240-4220)	1.2 (1.1-1.4)	Depression, porphyrin-like deposit around eyes, salivation, bloody urine, rough fur D.T. 4 hr-8 days
phthyl isobutyl ether		Rat	5930 (4750-7420)	1.9 (1.4-2.5)	Wet posterior, coma within 1 hr, rough fur and black, soft stools D.T. 1-6 days
zaldehyde		Rat	1300 (1110-1540)	1.4 (1.2-1.6)	Depression, coma on higher doses D.T. 4-18 hr
do.		Guinea-pig	1000 (800-1250)	1.4 (1.2-1.8)	Diuresis, tremors, intestinal irritation and haemorrhage D.T. 1 hr-4 days
zene†		Rat	4080 (3260-5100)	1.7 (1.4-2.1)	Tremors, loss of equilibrium and comatose on high doses, scrawny appearance for several days D.T. 1 hr-4 days
zyl acetate		Rat	2490 (2040-3040)	1.6 (1.1-2.4)	Depression D.T. 4 hr-3 days
zyl alcohol		Rat	1230 (1130-1330)	1.2 (1.1-1.3)	Depression, coma within 10-15 min. Excitable for 3-4 days D.T. 1 hr-4 days
do.	A-25%	Mouse	1580 (1410-1770)	1.2 (1.1-1.3)	Depression D.T. 2-18 hr
zyl n-butyrate		Rat	2330 (1940-2800)	1.3 (1.1-1.6)	Depression, scrawny appearance, tremors with higher doses D.T. 4 hr-4 days
benzyl butanoate)					
zyl cinnamate		Rat	5530 (3100-7740)	2.2 (0.9-3.6)	Depression, coma persisting in some rats for 24 hr D.T. 4 hr-5 days
innamein, benzyl phenylacrylate)		Guinea-pig	3760 (2340-6055)	4.2 (1.3-13.8)	Depression, gastro-intestinal tract irritation, rectal bleeding D.T. 4 hr-6 days
do.					
utyl alcohol		Rat	2510 (2220-2840)	1.4 (1.2-1.7)	Depression, coma D.T. 4-18 hr
utanol)					

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
put oil		Rat	3870 (3360-4450)	1.2 (1.1-1.3)	Scrawny appearance, wet posterior. Gross pathology shows pale, nutmeg livers. Depression, persisting in some animals for as long as 3 days D.T. 4 hr-9 days
amus oil		Rat	777 (612-987)	1.7 (1.3-2.3)	Severe tremors from 30 min-2 hr after treatment. Scrawny appearance, weight loss for several days D.T. 4 hr-8 days
aprylic acid		Rat	10,080 (8190-12,370)	1.6 (1.3-2.0)	Depression, diarrhoea D.T. 4 hr-9 days
octanoic acid)		Rat	810 (710-920)	1.2 (1.1-1.3)	Depression within 10 min, coma within 1 hr D.T. 1 hr-3 days
vacrol					
2-methyl-5-isopropylphenol)		Rat	1640 (1260-2130)	1.8 (1.3-2.5)	Depression, ataxia (loss of use of hind legs) D.T. 4 hr-5 days
vone					
7-mentha-6, 8-dien-2-one)		Guinea-pig	766 (603-845)	1.3 (1.1-1.6)	Extreme depression D.T. 4-18 hr
do.					
eryl acetate		Rat	44,750 (33,650-59,520)	1.6 (1.3-2.0)	Depression, scrawny appearance, rough, wet fur D.T. 4 hr-11 days
namic aldehyde		Rat	2220 (1910-2600)	1.4 (1.2-1.6)	Depression, diarrhoea, scrawny appearance D.T. 2-3 hr
aldehyde)					
do.		Guinea-pig	1160 (955-1420)	1.5 (1.2-1.9)	Coma with higher doses D.T. 2 hr-4 days
aconic acid	B-25%	Rat	1320 (1070-1640)	1.6 (1.1-2.4)	Depression, scrawny appearance, tremors, stomach haemorrhage D.T. 4 hr-3 days
is-methylbutenedioic acid)					
do.	B-25%	Mouse	2260 (1930-2640)	1.4 (1.2-1.6)	Depression, laboured respiration, haemorrhage in gastro-intestinal tract D.T. few min-3 days



Table 1 (Continued)

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Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.	B—25%	Guinea-pig	1350 (1040-1755)	1.9 (1.1-3.1)	Scrawny appearance, gastro-intestinal tract irritation D.T. few min-3 days
do.		Rat	4960 (3940-6240)	1.5 (1.2-2.0)	Depression D.T. 4 hr-4 days
1,1-dimethyl-2,7-octadiene	A—5%	Rat	680 (505-920)	1.8 (1.0-3.5)	Depression soon after treatment, mottled livers D.T. 4 hr-8 days
2,3-dihydro-4H-benzopyran-2-one	C†—10%	Guinea-pig	202 (179-228)	1.2 (1.1-1.4)	Depression, ataxia, severe gastro-intestinal tract irritation D.T. 1-6 days
do.		Rat	1390 (1140-1700)	1.5 (1.3-1.8)	Depression within 1 hr after treatment, scrawny appearance, porphyrin-like deposit around eyes and nose. Gross pathology showed discoloured liver, irritated gastro-intestinal tract, stomach haemorrhage, yellowish material attached to the intestines D.T. 4 hr-10 days (most deaths within 2 days)
3-methyl-2-phenylacetaldehyde		Rat	3810 (3080-4730)	1.7 (1.3-2.2)	Ataxia soon after treatment, coma within 1 hr. Wet fur, porphyrin-like deposit around eyes and nose D.T. 1-6 days
2-methyl-3-phenylacetaldehyde		Rat	4750 (3720-6060)	1.7 (1.5-2.0)	Depression soon after dosing, coma, bloody lacrimation, diarrhoea. Irritable, scrawny appearance for as long as 2 weeks D.T. 4 hr-12 days
2-methyl-3-phenylacetaldehyde		Rat	1580 (1310-1920)	1.5 (1.2-1.9)	Depression followed by convulsions within 10-15 min after treatment D.T. few min-2 hr

†C=Propylene glycol

acceptable for food use because of toxic effects observed on chronic feeding.

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.		Guinea-pig	990 (728-1350)	2.4 (1.2-4.6)	Ataxia, gasping, coma D.T. few min-4 days
2-methylacetic acid†	B—2%	Rat	570 (467-695)	1.6 (1.3-1.9)	Depression soon after treatment, weight loss, scrawny appearance D.T. 3-5 days
2-methylacetic acid		Rat	4400 (3380-5720)	1.9 (0.6-5.6)	Depression, wet posterior D.T. 4 hr-3 days
1-methoxy-4-propylbenzene		Mouse	7300 (5930-9000)	1.6 (1.4-1.8)	Porphyrin-like deposit around eyes and nose, rough fur, scrawny appearance D.T. 1-4 days
do.		Rat	1460 (1180-1820)	1.6 (1.0-2.6)	Depression D.T. 2 hr-2 days
2-methylacetic acid		Guinea-pig	1760 (1460-2170)	1.6 (1.2-2.1)	Depression D.T. 4 hr-4 days
2-methylacetic acid		Rat	2260 (1840-2780)	1.7 (1.4-2.0)	Depression soon after treatment, scrawny appearance, lacrimation D.T. 2-5 days
2-methylacetic acid		Mouse	4300 (3420-5420)	1.8 (1.2-2.7)	Severe ataxia, coma D.T. 4 hr-5 days
2-methylacetic acid		Rat	1280 (934-1770)	2.3 (1.0-5.1)	Depression, coma D.T. 1-24 hr
do.		Guinea-pig	988 (705-1380)	1.7 (1.4-2.0)	Diuresis, coma. Severe gastro-intestinal tract irritation D.T. 1 hr-4 days
2-methylacetic acid	B—2%	Rat	670 (598-750)	1.2 (1.0-1.4)	Depression, scrawny appearance, diarrhoea in 1 hr D.T. 2 hr-2 days
2-methylacetic acid		Rat	924 (825-1030)	1.2 (1.1-1.3)	Depression, abdominal cavity filled with bloody fluid D.T. 2 hr-5 days
2-methylacetic acid		Rat	13,050 (12,210-13,940)	1.2 (1.0-1.3)	Depression within a few min, coma on higher doses D.T. 4-18 hr

Table 1 (Continued)

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Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
1-methyl-2-ethylhexanoate		Rat	25,950 (22,190-30,170)	1.4 (1.2-1.6)	Depression, coma, wet posterior D.T. 4 hr-4 days
do.		Rat	1850 (1520-2210)	1.6 (1.3-1.9)	Depression within 5-10 min Laboured respiration D.T. 15 min-2 hr
do.		Guinea-pig	1110 (887-1190)	2.0 (1.3-3.1)	Depression, irritated gastro-intestinal tract D.T. 10 min-2 hr
1-methylphenyl glycidate (ethyl ester of 2,3-epoxy-methyl-3-phenylpropionate)		Rat	5170 (4670-6410)	1.3 (1.1-1.5)	Depression, rough fur, porphyrin-like deposit around eyes and nose D.T. 4 hr-8 days
do.		Guinea-pig	4050 (3540-4620)	1.5 (1.2-1.8)	Depression in varying degrees, rectal bleeding on high doses D.T. 4 hr-7 days
1-oenanthate (ethyl heptanoate)		Rat	>34,640*		Depression, coma, rough and wet fur
1-oxyhydrate†		Rat	14,700 (13,300-16,240)	1.1 (0.9-1.4)	Coma within 5 min D.T. 4 hr-5 days
1-pelargonate (ethyl nonanoate)		Rat	>43,000*		Depression, irritated gastro-intestinal tract. Appeared normal 24 hr after treatment
do.		Guinea-pig	24,190 (19,350-30,240)	1.5 (1.2-2.0)	Depression, irritated gastro-intestinal tract D.T. 4 hr-6 days
1-sebacate (ethyl decadioate)		Rat	14,470 (12,805-16,350)	1.4 (1.1-1.5)	Wet fur, depression D.T. 4 hr-4 days
do.		Guinea-pig	7280 (5970-8900)	1.3 (1.1-1.4)	Depression, diuresis D.T. 8 hr-6 days
1-vanillin (2-ethoxy-4-hydroxy-benzaldehyde)	C-20%	Rat	>2000**		Depression, coma on high doses D.T. 3-18 hr

†Ethyl oxyhydrate is a mixture whose composition varies with the method of manufacture. The material used in this study was obtained from Florasynth Labs., Inc., N.Y.

\*\*Toxicity of the propylene glycol solvent prohibited administration of higher doses.

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
1-allyptol (1,8-epoxy-p-menthane)		Rat	2480 (2100-2930)	1.4 (1.2-1.6)	Depression, coma on high doses, scrawny appearance for 3-4 days. Recovery within 7 days D.T. 2 hr-4 days
1-phenol (1-hydroxy-2-methoxy-4-allylbenzene)		Rat	2680 (2420-2970)	1.2 (1.1-1.4)	Coma soon after treatment D.T. approximately 1 hr
do.		Mouse	3000 (2400-3750)	1.8 (1.3-2.3)	Severe depression immediately after treatment D.T. few min-2 days
do.		Guinea-pig	2130 (1860-2430)	1.3 (1.2-1.5)	Depression D.T. 4 hr-3 days
1-phenol acetate (1-acetoxy-2-methoxy-4-allylbenzene)		Rat	1670 (1265-2200)	1.9 (1.4-2.5)	Rough fur, depression D.T. 4 hr-2 days
1-phenol methyl ether (1,2-dimethoxy-4-allylbenzene)		Rat	1560 (1170-2070)	2.6 (1.6-4.3)	Coma within 1 hr after treatment D.T. less than 8 hr
1-methone (7,1,3,3-trimethyl-2-orecamphanone)		Rat	6160 (4400-8630)	2.7 (1.1-6.5)	Depression, scrawny appearance, porphyrin-like deposit around eyes and nose for a week after treatment D.T. 4 hr-9 days
1-uraldehyde	A-5%	Rat	127 (110-147)	1.3 (1.2-1.5)	Depression soon after treatment. Scrawny appearance, porphyrin-like deposit around eyes and nose. Gross pathology showed lung haemorrhage on the higher doses D.T. 4 hr-4 days
1-raniol extra (3,7-dimethyl-2,6-octadienol)		Rat	3600 (2840-4570)	1.7 (1.3-2.2)	Depression, coma, wet fur D.T. 4-18 hr
1-raniol (3,7-dimethyl-1,6-octadienol)		Rat	6330 (5450-7340)	1.3 (1.2-1.4)	Depression, coma D.T. 4 hr-3 days
1-raniol butyrate		Rat	10,660 (8020-14,180)	1.6 (0.7-3.7)	Marked depression and coma on higher doses

Table 1 (Continued)

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Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
iac gum	A—20%	Rat	>5000*	—	Rough fur, scrawny appearance. D.T. less than 18 hr
		Rat	3270 (2780-3830)	1.6 (1.3-2.0)	Coma on higher doses within 30 min. Scrawny appearance, rough fur, porphyrin-like deposit around eyes and nose
ratropic aldehyde -phenylpropanal)		Rat	2800 (2390-3280)	1.3 (1.2-1.4)	D.T. 1-4 days Coma within 20 min after treatment. Rough fur
one 0% $\alpha$ -ionone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-buten-2-one and 40% ionone, 4-(2,6,6-trimethyl-cyclohexen-1-yl)-3-meten-2-one]		Rat	4590 (3880-5400)	1.4 (1.3-1.5)	D.T. 1-5 days Depression, tremors D.T. 4 hr-4 days
myl formate		Rat	9840 (7720-12,550)	1.7 (1.3-2.3)	Depression soon after treatment, scrawny appearance D.T. 4 hr-4 days
ugenol -hydroxy-2-methoxy-4-propenylbenzene)		Rat	1560 (1290-1880)	1.4 (1.3-1.5)	Coma soon after treatment, scrawny appearance D.T. 1 hr-7 days
do.		Guinea-pig	1410 (1130-1780)	2.0 (1.5-2.7)	Depression, coma D.T. 3-6 days
afrole§ 2-methylenedioxy-4-propenylbenzene)		Rat	1340 (1140-1590)	1.4 (1.2-1.7)	Depression, coma, rough fur, scrawny appearance D.T. 4 hr-8 days
do.	A—25%	Mouse	2470 (2010-3040)	1.7 (1.3-2.2)	Depression soon after treatment. Severe ataxia within 24 hr. Recovery from ataxia but scrawny in appearance within 48 hr D.T. 1 hr-4 days

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
per tar		Rat	8014 (6550-9770)	1.5 (1.2-1.9)	Depression soon after treatment, scrawny appearance for several days. Irritated gastro-intestinal tract D.T. 4 hr-4 days
llool 7-dimethyl-1,6-octadien-ol or 3,7-dimethyl-1,7-octadien-3-ol)		Rat	2790 (2440-3180)	1.3 (1.2-1.4)	Ataxia soon after treatment D.T. 4-18 hr
yl acetate		Rat	14,550 (12,300-17,170)	1.8 (1.3-2.6)	Depression soon after treatment, coma, wet posterior D.T. 4 hr-4 days
do.		Mouse	13,360 (11,920-15,000)	1.2 (1.1-1.3)	Depression within 10-15 min after treatment D.T. 1-3 days
yl cinnamate		Rat	9960 (8230-12,050)	1.4 (1.2-1.7)	Depression. Scrawny appearance, porphyrin-like deposit around eyes and nose, wet fur on posterior 24 hr after treatment D.T. 4 hr-5 days (most deaths in 48 hr)
yl isobutyrate		Rat	>36,300*	—	Depression, wet fur, diarrhoea. Appeared normal after 1 week
do.		Mouse	15,100 (12,330-18,500)	1.6 (1.1-2.2)	Depression soon after treatment. Excitable after 1 hr. Rough fur D.T. 4 hr-3 days
ce oil		Rat	3640 (3170-4190)	1.4 (1.0-2.0)	Depression. Scrawny appearance for several days after treatment D.T. 4-18 hr
ilotic anhydride anhydride of <i>o</i> -hydroxy-namic acid)		Rat	1510 (1310-1750)	1.3 (1.1-1.6)	Depression D.T. 1 hr-2 days
thol -menthan-3-ol)	A—50%	Rat	3180 (2790-3620)	1.3 (1.1-1.5)	Ataxia, scrawny appearance D.T. 4 hr-3 days

Table 1 (Continued)

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Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
1-anthranilate benzoic acid, (r)		Rat	2910 (2500-3400)	1.4 (0.9-2.0)	Depression, coma D.T. 1-2 days
do.		Mouse	3900 (3260-4680)	1.5 (1.3-1.7)	Depression D.T. 4-18 hr
do.		Guinea-pig	2780 (2210-3500)	1.8 (1.4-2.3)	Depression, gasping, rapid respiration, irritated gastro-intestinal tract D.T. 4 hr-4 days
yl benzoate		Rat	1350 (1290-1410)	1.1 (0.7-1.5)	Depression, porphyrin-like deposit around nose, rough fur, wet posterior. Survivors excitable D.T. 2-18 hr
do.	A-50%	Mouse	3330 (2920-3800)	1.2 (1.1-1.4)	Excitation, tremors D.T. few min-18 hr
ylenedioxybenzene†		Rat	580 (487-690)	1.4 (1.2-1.7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1-6 days
do.	A-25%	Mouse	1220 (976-1520)	1.4 (1.2-1.7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1-5 days
yl salicylate hydroxybenzoic acid, (thyl ester) do.		Rat	887 (715-1100)	1.5 (1.2-1.8)	Depression soon after treatment D.T. 4-18 hr
		Guinea-pig	1060 (873-1300)	1.6 (1.3-1.9)	Convulsions. Irritated gastro-intestinal tract D.T. 1 hr-3 days
ak ambrette (artificial) tert-butyl-3-methyl-2, dinitroanisole)	A-25%	Rat	339 (283-408)	1.4 (1.2-1.7)	Increased respiration and hypersensitivity 24 hr after treatment. Scrawny appearance, wet posterior, rough fur D.T. 1-3 days

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
neg oil		Rat	2620 (2200-3120)	1.3 (1.2-1.5)	Depression soon after receiving treatment, scrawny appearance for several days D.T. 4-18 hr
ylethyl alcohol phenylethanol)		Rat	1790 (1580-2020)	1.2 (1.1-1.3)	Coma within 15 min. Gross pathology shows irritation of the lower half of the stomach on the higher doses D.T. 4-18 hr
ylethyl phenylacetate		Rat	15,390 (12,830-18,470)	1.5 (1.4-1.7)	Depression soon after treatment, scrawny appearance for several days D.T. 4 hr-5 days
onal 4-methylenedioxy-izaldehyde) panol	A-25%	Rat	2700 (2350-3100)	1.5 (1.1-2.0)	Tremors for several hr followed by depression and ataxia D.T. 2 hr-5 days
		Rat	6500 (5800-7280)	1.2 (1.1-1.3)	Coma soon after treatment, scrawny appearance D.T. 2-18 hr
enylbenzene†		Rat	3600 (2650-4900)	2.2 (1.3-3.8)	Scrawny appearance, weight loss, wet posterior for 5-7 days after treatment D.T. 4 hr-9 days
pyl acetate		Rat	9370 (7670-11,430)	1.5 (1.1-1.8)	Depression soon after treatment, rough fur, scrawny appearance D.T. 4-18 hr
do.		Mouse	8300 (7280-9160)	1.2 (1.1-1.4)	Depression soon after treatment D.T. few min-18 hr
ylbenzene†		Rat	6040 (4830-7550)	1.5 (1.2-2.0)	Depression soon after receiving treatment. Scrawny appearance D.T. 1-3 days
pyl n-butyrate		Rat	15,000 (12,600-17,850)	1.4 (0.8-2.5)	Depression soon after treatment followed by coma on higher doses. Rough fur, diarrhoea D.T. 1-3 days
pyl formate		Rat	3980	1.5	Depression soon after treatment

ACUTE ORAL TOXICITY OF FOOD FLAVOURINGS

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.		Mouse	3400 (3060-3780)	1.7 (1.3-2.2)	Depression soon after treatment D.T. few min-6 hr
4-ethyl-2-methylenedioxy-4-ylbenzene)		Rat	1950 (1760-2160)	1.3 (0.6-3.0)	Depression, ataxia, diarrhoea D.T. 1-5 days
do.	A-50%	Mouse	2350 (2010-2750)	1.4 (1.1-1.6)	Ataxia, depression, scrawny appearance
vinyl acetate menth-1-en-8-ol ester acetic acid)		Rat	5075 (4160-6190)	1.5 (1.3-1.7)	D.T. 4 hr-7 days Depression, scrawny appearance, porphyrin-like deposits around eyes and nose
ool	C-20%	Rat	980 (817-1180)	1.6 (1.3-2.0)	D.T. 4 hr-5 days Depression, ataxia, coma on high doses
cymen-3-ol,3-droxy-p-cymene)	C-20%	Guinea-pig	880 (740-1050)	1.6 (1.2-2.1)	D.T. 4 hr-5 days Irritated gastro-intestinal tract, tremors, coma, respiratory failure
do.					D.T. 1 hr-10 days
ilin	C-20%	Rat	1580 (1390-1810)	1.3 (1.2-1.5)	Coma soon after treatment D.T. 4 hr-4 days
hydroxy-3-methoxy-nzaldehyde)		Guinea-pig	1400 (1310-1500)	1.1 (1.09-1.2)	Depression within 1 hr D.T. 1-3 days
do.		Rat	1360 (980-1870)	1.6 (1.3-1.9)	Coma within 10 min after treatment. Salivation, porphyrin-like deposit around eyes, diarrhoea, scrawny appearance for 3-4 days D.T. 1-4 days
role	A-25%	Mouse	2020 (1650-2480)	1.7 (1.4-2.0)	Rapid, laboured respiration; lacrimation; pawing about mouth; hyperactivity followed by coma within 15 min D.T. few min-4 hr
2-dimethoxybenzene)					

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## Condiments et Complexes de Structure Voisine. I. Toxicité Aiguë par Voie Buccale

Résumé—On administra par intubation des doses de complexes faits de 107 condiments synthétiques et naturels, de structure chimique voisine, à des souris, des rats et des cobayes. On observa habituellement les animaux pendant 2 semaines, durant lesquelles on suivit le développement de signes toxiques et on nota la date de la mort. Pour chaque complexe on détermina la dose orale limite au-delà de laquelle commence l'intoxication aiguë.

Lebensmittelgeschmackszusätze und Verbindungen verwandter Strukturen  
I. Akute Oraltoxität

Zusammenfassung—107 synthetischen und natürlichen Geschmackszusätze und strukturverwandte Verbindungen wurden durch Intubation an Mäuse, Ratten und Meerschweinchen verabreicht. Die Tiere wurden gewöhnlich 2 Wochen lang unter Beobachtung gehalten, während welcher Zeit die Entwicklung toxischer Symptome verfolgt und die Zeit des Todes eintritts registriert wurde. Die akute orale mittlere tödliche Dosis jeder Verbindung wurde festgestellt.

Table 1 (Continued)

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Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
1 anthranilate benzoic acid, (ester)		Rat	2910 (2500-3400)	1.4 (0.9-2.0)	Depression, coma D.T. 1-2 days
do.		Mouse	3900 (3260-4680)	1.5 (1.3-1.7)	Depression D.T. 4-18 hr
do.		Guinea-pig	2780 (2210-3500)	1.8 (1.4-2.3)	Depression, gasping, rapid respiration, irritated gastro-intestinal tract D.T. 4 hr-4 days
yl benzoate		Rat	1350 (1290-1410)	1.1 (0.7-1.5)	Depression, porphyrin-like deposit around nose, rough fur, wet posterior. Survivors excitable D.T. 2-18 hr
do.	A-50%	Mouse	3330 (2920-3800)	1.2 (1.1-1.4)	Excitation, tremors D.T. few min-18 hr
ylenedioxybenzene†		Rat	580 (487-690)	1.4 (1.2-1.7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1-6 days
do.	A-25%	Mouse	1220 (976-1520)	1.4 (1.2-1.7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1-5 days
yl salicylate hydroxybenzoic acid, (ethyl ester)		Rat	887 (715-1100)	1.5 (1.2-1.8)	Depression soon after treatment D.T. 4-18 hr
do.		Guinea-pig	1060 (873-1300)	1.6 (1.3-1.9)	Convulsions. Irritated gastro-intestinal tract D.T. 1 hr-3 days
ak ambrette (artificial) tert-butyl-3-methyl-2, 6-dinitroanisole)	A-25%	Rat	339 (283-408)	1.4 (1.2-1.7)	Increased respiration and hypersensitivity 24 hr after treatment. Scrawny appearance, wet posterior, rough fur D.T. 1-3 days

Table 1 (Continued)

Compound	Concentration (w/v) and solvent used	Species	LD <sub>50</sub> with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
neg oil		Rat	2620 (2200-3120)	1.3 (1.2-1.5)	Depression soon after receiving treatment, scrawny appearance for several days D.T. 4-18 hr
ylethyl alcohol phenylethanol)		Rat	1790 (1580-2020)	1.2 (1.1-1.3)	Coma within 15 min. Gross pathology shows irritation of the lower half of the stomach on the higher doses D.T. 4-18 hr
ylethyl phenylacetate		Rat	15,390 (12,830-18,470)	1.5 (1.4-1.7)	Depression soon after treatment, scrawny appearance for several days D.T. 4 hr-5 days
onal 4-methylenedioxy-benzaldehyde) spanol	A-25%	Rat	2700 (2350-3100)	1.5 (1.1-2.0)	Tremors for several hr followed by depression and ataxia D.T. 2 hr-5 days
nylbenzene†		Rat	6500 (5800-7280)	1.2 (1.1-1.3)	Coma soon after treatment, scrawny appearance D.T. 2-18 hr
pyl acetate		Rat	3600 (2650-4900)	2.2 (1.3-3.8)	Scrawny appearance, weight loss, wet posterior for 5-7 days after treatment D.T. 4 hr-9 days
do.		Rat	9370 (7670-11,430)	1.5 (1.1-1.8)	Depression soon after treatment, rough fur, scrawny appearance D.T. 4-18 hr
ylbenzene†		Mouse	8300 (7280-9160)	1.2 (1.1-1.4)	Depression soon after treatment D.T. few min-18 hr
		Rat	6040 (4830-7550)	1.5 (1.2-2.0)	Depression soon after receiving treatment. Scrawny appearance D.T. 1-3 days
pyl n-butyrate		Rat	15,000 (12,600-17,850)	1.4 (0.8-2.5)	Depression soon after treatment followed by coma on higher doses. Rough fur, diarrhoea D.T. 1-3 days
pyl formate		Rat	3980	1.5	Depression soon after treatment

Katsumata, Kazuo, and A. Niki: METABOLISM OF ACETOIN AND DIACETYL IN DIABETIC ANIMALS. *Nippon Naibumpi Gakkai Zasshi*, Vol. 36, pp. 1635-41, 1960.  
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(Director: Prof. K. Yamada).

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## I. INTRODUCTION

Neuberg and his collaborators reported that acetoin was formed during the process of fermentation of yeast sucrose or pyruvic acid, and named the enzyme carbolligase (1,2). Later, acetoin was found in human urine (3,4), and also in pig heart (Green et al. (5)), rat wuchereria (Bueding et al.) (6), and cow wuchereria (Yonezawa) (7,8), which led to the general belief that this enzyme is metabolized in the system of a wide variety of animals (9-11). The purification of this enzyme is yet to be perfected (12). In addition to the reaction of pyruvic acid, or pyruvic acid and acetaldehyde in the presence of a coenzyme of T.P.P.,  $Mg^{++}$  (13), or  $Mn^{++}$ , acetoin is formed by way of a reaction producing various betols (14,15). How the resultant acetoin is metabolized *in vivo* is yet to be clarified, but it is known that a part of it is excreted in the original form and the rest in the form of diacetyl, 2,3 butyleneglycol (16,3,4). Two molecules of diacetyl becomes 2 molecules of acetaldehyde and 1 molecule of acetoin in the presence of diacetyl mutase (17) and T.P.P., through the reaction of which the enzyme relates to the T.G.A. cycle. Mizuhara postulated the following route as representing the relationship between pyruvate, citrate, and acetoin, based on the results of his experiment using rabbits.

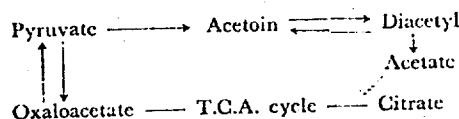


Figure 1

On the basis of their observation that diacetyl was oxidized by a sample of pyruvic oxidase (19,20) although the reaction was slow, Schweet et al. maintained that diacetyl mutase is identical to pyruvic oxidase (21). The physiological significance of acetoin is totally unknown. Diacetyl, the oxidation product of acetoin, is transformed into acetaldehyde and an acetaldehyde-enzyme complex by diacetyl mutase. This complex is regarded as identical to the acetaldehyde enzyme complex produced from pyruvate (31). Therefore, diacetyl may be used as an acetyl donor *in vivo*, and a few experiments have actually demonstrated the fact (22-25). Akabori et al. stated that acetoin  $\rightleftharpoons$  diacetyl forms an oxidation reduction system, but, in a later experiments using  $C^{14}$ -labelled acetaldehyde, they confirmed that it was simply transcarbolligation (26,27). Dwanson et al. extracted the acetoin forming system from pig heart by the method employed by Korkes et al. (28), and utilized it in various experiments, in which they observed that pyruvate became active acetaldehyde by decarboxylation, subsequently transformed into acetoin in the presence of an acceptor, i.e., acetaldehyde, and acetyl CoA in the presence of lipoic acid. Whether acetoin is produced or acetyl CoA is formed depends upon the availability of the enzyme of cofactor (29). Meanwhile, Dwanson et al. incubated a homogenate of rabbit

kidney with acetoin, and obtained acetoacetic acid (30). Mizuhara also incubated a homogenate of rat liver with diacetyl, and obtained acetoacetate (31). The behavior of acetoin and diacetyl in diabetes is totally unknown. We assumed that in diabetes, the production of acetoin becomes excessive due to a drop in CoA, lipoic acid, or T.P.P., and its decomposition is also retarded. Based on this assumption, we carried out a few experiments.

## II. EXPERIMENTAL PROCEDURES

### QUANTITATIVE DETERMINATION, AND DISCUSSION

#### 1. QUANTITATIVE DETERMINATION OF ACETOIN

Acetoin can be determined by the gravimetric method (32), polarographic method (33), and the most sensitive, colorimetric method (34-38), but Westerfeld's method was employed in this experiment (35). The entire acetoin was transformed into diacetyl, and the diacetyl thus obtained and the diacetyl in the sample were subjected to the reaction in which they displayed a red coloration in the presence of creatine,  $\alpha$ -naphthol, and alkali. Since acetoin and diacetyl are expressed as acetoin inclusively, some call the combination the total acetoin. For the purification of acetoin by means of a standard curve, commercial acetoin was permitted to crystallize in a refrigerator for approximately 10 days, and a 25 g portion of it was washed with 40 cc of acetone, which was subsequently eliminated under reduced pressure. This procedure was repeated 3 times, which produced white crystals. The  $\alpha$ -naphthol used for the measurement was purified by steam distillation in a flow of  $N_2$  gas. Ikuno (39) gave a detailed account on a care to be excersized during the measurement procedure.

#### 2. QUANTITATIVE DETERMINATION OF ACETALDEHYDE

The serum acetaldehyde level was determined according to Stotz's method (40).

#### 3. XANTHINE OXIDASE ACTIVITY MEASUREMENT

Dhungat's method was employed for the measurement of liver xanthine oxidase activity.

### EXPERIMENTAL PROCEDURE

Male, white Wistar rats weighing approximately 90 g were used. They were given mixed feed (ground white onion, powdered rice, bread crumbs, fish powder) and vegetable for several days. For the alloxan-treated group, alloxan was intraperitoneally injected at 200 mg per kg, and only those which showed the urine sugar level higher than ++ by testape (unable to verify) 72 hours and 1 week later, were used for the experiment. The vitamin  $B_1$  deficiency group consisted of rats which were given the basal composite feed normally used in this laboratory from which  $B_1$  had been eliminated, and the control group, the basal composite feed. A group of rats were given a smaller amount of feed so that their body weight remained at the same level as that of the  $B_1$  deficiency group. The blood, liver, kidney, and urine acetoin levels before treatment and after acetaldehyde injection, and the blood acetaldehyde level after the administration of acetaldehyde of each group were measured. The liver xanthine oxidase activity was also measured. The above experiments were carried out after 4 hours of starvation. The healthy and diabetic human subjects were withheld from any injection or drug from the day before the



# Liver Acetoin Level of Alloxan-Treated White Rats

Alloxan-treated white rats and control rats were phlebotomized by decapitation, and a liver homogenate was obtained from 3 cc of distilled water and 3 g of liver. A 4 cc portion of the homogenate was subjected to measurement. In terms of the amount of acetoin per 4 g of liver, there was no difference between the two groups, as noted in Table 1.

## Liver Acetoin Level Following the Administration of Acetaldehyde

Forty-five mg per 100 g of acetaldehyde was injected into the abdominal cavity. The rats were decapitated 5, 10, and 15 minutes later, and their liver acetoin levels were examined. An increase was shown by both groups, but as noted in Figure 4, the values of the alloxan-treated rats were considerably lower.

TABLE 1

a 例数	b 体重	c 種類	d 肝 acetoin 値 7/4g	e 平均値
7	90	72h Alloxan	0.45, 0.39, 0.32, 0.22, 0.12, 0.30, 0.34	0.30
7	92	control	0.44, 0.38, 0.32, 0.28, 0.44, 0.12, 0.30	0.33

KEY: a, number of cases; b, body weight; c, group; d, liver acetoin value; e, mean value

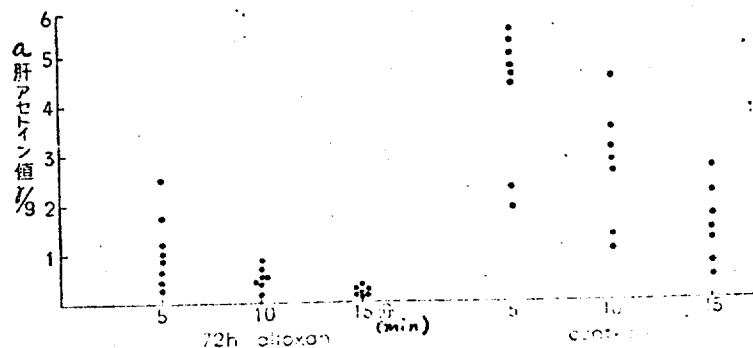


Figure 4 (Key: a, liver acetoin value)

## Kidney Acetoin Level of Alloxan-Treated White Rats

The animals of both groups were phlebotomized by decapitation, and their kidneys removed. A kidney homogenate was prepared from 1 g of the organ and 3 cc of distilled water. A 3 cc portion of the paste was subjected to quantitative determination of acetoin. In terms of the amount of acetoin per 4 g of kidney, the data shown in Table 2 were obtained. No difference was noted between the groups.

TABLE 2

a 例数	b 体重	c 種類	d 腎 acetoin 値 7/4g	e 平均値
7	90	72h alloxan	0.95, 0.92, 0.80, 1.20, 1.0, 0.99	0.98
7	92	control	0.82, 1.8, 0.12, 0.95, 0.8, 1.0	0.91

KEY: a, number of cases; b, body weight; c, group; d, kidney acetoin value; e, mean value

# Kidney Acetoin Level Following the Administration of Acetaldehyde

Acetaldehyde was injected as in the previous experiment, and the kidney acetoin level was measured 5, 10, and 15 minutes later. The alloxan-treated white rats showed significantly higher values as shown in Figure 5.

## 3. ACETOIN CONCENTRATION IN THE URINE OF ALLOXAN-TREATED WHITE RATS

Many papers have been written on the acetoin concentration in human urine (44, 3, 4). Miyaji et al. gave healthy human individuals 0.6 g of 1 NAH per day for 5 days, and observed an increase in the concentration of urine diacetyl. When Ca pantothenate was hypodermically injected to the subjects at 10 mg per day for 5 days, the concentration dropped considerably (45). The white rats of the two groups were placed in metal mesh chamber, and given water only, without feed, and 12-hour urine was collected for the determination of acetoin concentration.

As shown in Figure 6, the alloxan-treated white rats gave considerably higher values.

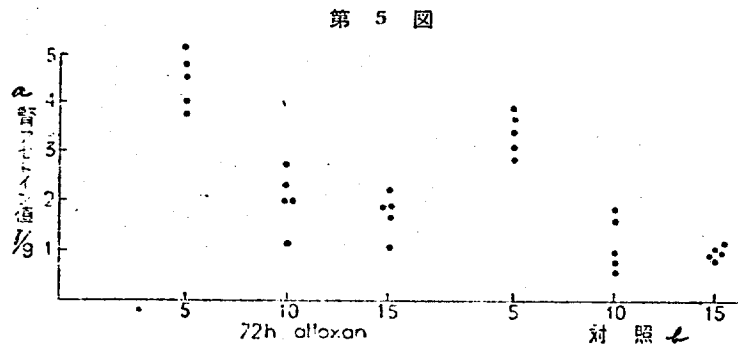


Figure 5 (KEY: a, kidney acetoin value; b, control)

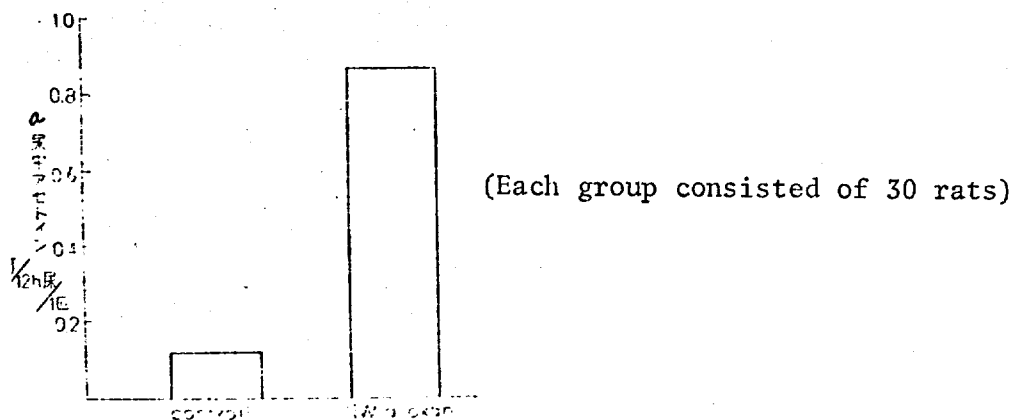


Figure 6. (Key: a, urine acetoin, 1/12 h urine/animal)

#### 4. THE ACETALDEHYDE CONCENTRATION IN THE BLOOD OF ALLOXAN-TREATED WHITE RATS FOLLOWING THE ADMINISTRATION OF ACETALDEHYDE

Acetaldehyde was intraperitoneally injected to the alloxan-treated white rats and the control animals at 45 mg per 100 g, and 1 cc of blood was drawn from their abdominal aorta 60 minutes later. The results of measurement are shown in Table 3. It was found that acetaldehyde was retained in the blood of alloxan-treated white rats longer than in the blood of the control.

Table 3

例数 <sup>a</sup>	体重 <sup>b</sup>	组别 <sup>c</sup>	血中 acetaldehyde 值 <sup>d</sup>	平均 <sup>e</sup>
6	93	72h alloxan	381. 342. 644. 608. 742. 404	483
6	84	control	372. 294. 542. 438. 412. 500	422

KEY: a, number of cases; b, body weight; c, group; d, blood acetaldehyde; e, mean value.

#### 5. LIVER XANTHINE OXIDASE ACTIVITY OF ALLOXAN-TREATED WHITE RATS

Horita et al. observed decreases in total liver B<sub>2</sub> (46), D amino acid oxidase activity (47), and xanthine oxidase activity (48) in alloxan-treated diabetic white rats.

The retardation in the decomposition of acetaldehyde in alloxan-treated white rats seemed to be caused primarily by the drop in xanthine oxidase activity. Under this assumption, the liver xanthine oxidase activity was examined by the modified Dhungat's method developed by Axelrod (49). In terms of 2-hour O<sub>2</sub>ul per g of liver, the results shown in Figure 7 were obtained. The values shown by the alloxan-treated white rats were lower than the control level.

#### 6. THE ACETOIN CONCENTRATION IN THE BLOOD OF DIABETIC PATIENTS

Ikuno et al. observed an increase in blood acetoin concentration in patients with cancer of the uterus. Dwanson et al. measured the blood acetoin concentration in paranoic patients and found an increase in value during the state of depression (50).

We measured the blood acetoin concentration in diabetic patients and healthy individuals, and confirmed that the concentration rose in diabetes as shown in Table 4.

Table 4

例数 <sup>a</sup>	组别 <sup>b</sup>	血中 acetoin 值 r/dl <sup>c</sup>	平均 <sup>d</sup>
13	糖尿病患者	9.8, 13.5, 15, 9.4, 30, 12, 9, 7.5, 11, 24	15.8
10	健康人	6.0, 7, 10.5, 4.8, 5.4, 3.8, 9.4	6.9

KEY: a, number of cases; b, group; c, blood acetoin value; d, mean; e, diabetic; f, healthy

#### IV. DISCUSSION

The metabolic process of the acetoin and diacetyl compounds has not yet been fully clarified. Recently, Juni et al. carried out a series of experiments using dove pectoral muscles, and published their new findings on the mutual conversion cycle of diacetyl and acetoin compounds. The cycle is outlined in Figure 8.

Meanwhile, Fritz et al. observed the formation of acetoin from pyruvate in a homogenate of mouse Ehrlich ascitus cells (55). In their experiment using C<sup>14</sup>-labelled acetoin, Goodwin et al. demonstrated by means of eremothecium ashybie (56) that C<sup>14</sup> entered the ring A of riboflavin.

TABLE 5

a 例数	b 体重	c 種類	d 血中 acetoid $\gamma$ /dl
7	88	e B <sub>1</sub> 欠乏群	14.2
7	148	f 対照群	6.8
7	90g	g 体重抑制群	7.0

KEY : a, number of cases; b, body weight;  
c, group; d, blood acetoid; e, B<sub>1</sub>  
deficiency group; g, weight control  
group.

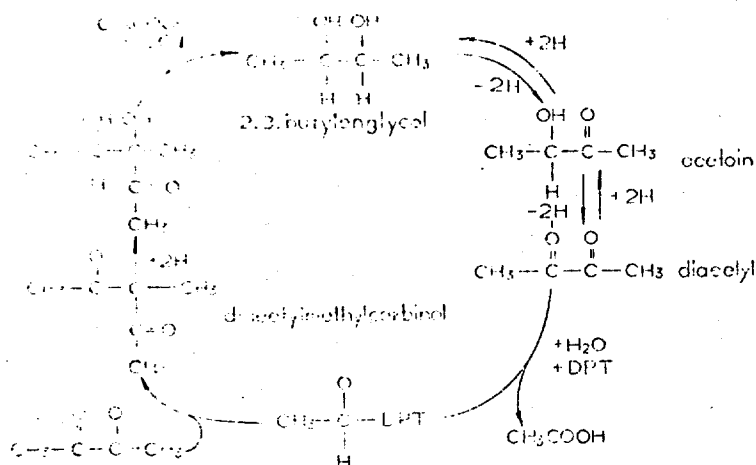


Figure 8

Martin et al. administered acetaldehyde in B<sub>1</sub>-deprived white rats, and examined the changes in the blood concentration of acetaldehyde. They found that the elimination of the compound from the blood was retarded as compared to that of the control, and the administration of acetoin resulted in an increase in the blood acetoin level of the B<sub>1</sub>-deprived rats, as compared to that of the control (57). Stotz et al. incubated a homogenate of rat brain with pyruvate and acetaldehyde, and observed subsequent formation of acetoin. They also reported that acetoin formation was delayed in B<sub>1</sub>-deprived rats (58). As already stated, B<sub>1</sub> is a coenzyme of carboligase and diacetyl mutase (59), and the behavior of acetoin at B<sub>1</sub> deficiency is of considerable interest. We

compared the blood acetoin levels of the control and the group of rats under weight control diet after 3 weeks of B<sub>1</sub> deprivation. The results, shown in Table 5, show that the values of the B<sub>1</sub> deprived group were sharply elevated.

Yamamura et al. examined the acetoin concentration in the blood of tuberculous patients, and found it to be slightly higher than normal, but failed to find any difference between tuberculous patients and patients with beriberi (60).

While raising mice on a pantothenic acid-deficient diet, Miyaji et al. found that their diacetyl level in vivo tripled or quadrupled. They attributed this to the disturbance of the physiological reaction in which diacetyl is changed into acetyl CoA, due to pantothenic acid deficiency (61).

It has been believed that CoA is not necessary for the production of acetoin (62), but Berry et al. maintained that CoA was necessary in an experiment in which acetoin powder of rat brain was employed (63).

The decrease in CoA, total B<sub>1</sub>, lipoic acid, and acetylation in alloxan-treated white rats have given a suitable condition for the acetoin concentration to rise.

The fact that the administration of acetaldehyde retarded the elimination of acetaldehyde from the blood in alloxan-treated white rats suggests a suppression of the liver xanthine oxidase activity.

The significance of the low liver acetoin value following the administration of acetaldehyde in alloxan-treated white rats is yet to be determined.

#### V. CONCLUSION

1. The blood and urine acetoin levels of the alloxan-treated white rats were higher than those of the control.
2. The blood acetaldehyde and acetoin levels of alloxan-treated white rats following the administration of acetaldehyde were elevated, but the liver acetoin concentration indicated an opposite tendency.
3. The blood acetoin values of diabetic patients are in many instances higher than the value of the healthy individuals.

(The authors are grateful to Prof. K. Yamada for his cooperation).

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# 糖尿病と Acetoin, Diacetyl 代謝

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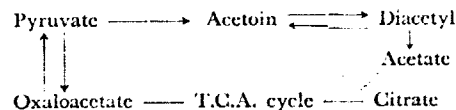
Nippon Naibonpi Gakkai Zasshi. 36: 1635-41 (1960)

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## I. 緒 言

Neuberg 及びその一門は, 酵母の糖質又は pyruvic acid の醗酵醗酵中に acetoin を生ずる事を報告し, 本酵素を carbolygase と名づけた<sup>1)</sup>。その後大塚<sup>2)</sup>にも acetoin の存在が確認された<sup>3)</sup>。更に Green 等は豚心臓に<sup>4)</sup>, Bueding 等は鼠糸状虫に<sup>5)</sup>, 米沢は牛糸状虫に<sup>6)</sup>本酵素の存在を認めるに及んで<sup>7)</sup>。広く動物組織で acetoin が代謝される事が明かとなった<sup>8-12)</sup>。本酵素の精製は尚不充分で<sup>13)</sup>, pyruvic acid, 又は pyruvic acid と acetaldehyde から T.P.P., Mg<sup>++</sup> 又は Mn<sup>++</sup> を<sup>14)</sup> 補酵素として acetoin を生ずる反応の他に, 種々の betol を生成する反応を触媒する<sup>15-17)</sup>。かくして生成された acetoin が生体内でどのように代謝されるかも尚不明であるが, 一部はそのままの形として, 或いは diacetyl, 2,3, butylenglycol として尿中に排泄される<sup>18) 19)</sup>。一方2分子の diacetyl は diacetyl mutase<sup>20)</sup>, T.P.P. により2分子の acetaldehyde と1分子の acetoin となり, この反応を通して T.C.A. cycle と連絡しているものと考えられている。水原は Pyruvate, citrate, acetoin との関係の点を明かした実験から第1図に示すごとく想定した<sup>21)</sup>。

第 1 図



一方 Schweet 等は diacetyl が pyruvic oxidase 標本によつて<sup>22) 23)</sup>, 反応速度はそれが酸化されるので, diacetyl mutase は pyruvic oxidase と同じものであるという興味ある報告をしている<sup>24)</sup>。尚 acetoin の生理的意義については殆んど知られていない。acetoin の酸化産物である diacetyl が diacetyl mutase により acetaldehyde と acetaldehyde-enzyme complex とに分れるが, この complex は pyruvate より生ずる acetaldehyde enzyme complex と同一のものであるとされている<sup>25)</sup>。従つて diacetyl は生体内で acetyl-donor として用いられることが考えられ, 事実これを確かめた<sup>2, 3</sup>の実験もある<sup>26-28)</sup>。赤堀等は acetoin と diacetyl が酸化還元系を形成すると述べたが, その後 C<sup>14</sup> labelled acetaldehyde を使つて, 単なる trans-carbolygation である事を確認した<sup>29) 30)</sup>。Dwanson 等は Korkes 等の方法により<sup>31)</sup> pig heart から acetoin 形成系を抽出し, これを用いて種々検討を加え, pyruvate が decarboxylation されて active acetaldehyde となり, acceptor として acetaldehyde があれば acetoin に, 又 lipoic acid があれば acetyl CoA が出来ると述べ, acetoin が出来るか, acetyl CoA となるかは, enzyme, cofactor の availability により影響されると説明している<sup>32)</sup>。一方 Dwanson 等は毛管臓の homogenate と acetoin を incubation し acetoacetic acid の生ずることを報告した<sup>33)</sup>。又, 水原も Rat の肝を homogenate し diacetyl と incubation すると acetoacetic acid が生ずるという<sup>34)</sup>。さて糖尿病時には見られる acetoin, diacetyl の糖質代謝では何れが原因でいえない。従つて, 糖尿病に際しては, CoA, lipoic acid, T.P.P. の減少等による acetoin の生成が増加し, しかもそれが連続するのではないかと考え, 以下の実験を行った。

## II. 実験方法

定量法, 並びにその検討

### 1. Acetoin の定量

acetoin の定量は, 大塚<sup>2)</sup>, 山田<sup>3)</sup>の方法を用いた方法<sup>35)</sup>で, 並びに最も鋭敏な比色法<sup>36) 37)</sup>があるが本実験には Westerfeld 法<sup>38)</sup>を用いた<sup>39)</sup>。方法は acetoin を酢素で diacetyl となし試料中の diacetyl と共に両者が creatine,  $\alpha$  naphthol, alkali のもとで紅色を呈する反応を利用したものである。Acetoin, diacetyl を含めて acetoin として測定するのを総 acetoin と呼ぶこともある。標準曲線を用いた acetoin の精製は市販の acetoin を 100 mg 程度で約10日間保存し結晶化し, その25 g を acetone 40cc で洗い, acetone を減圧除去する。これを3回くりかえして白色の結晶を得る。又測定に用いた  $\alpha$  naphthol は N<sub>2</sub>-gas のもとで水蒸気蒸留を行い精製した。測定上の注意としては山田<sup>3)</sup>が詳細に検討している。

### 2. Acetaldehyde の定量

血中 acetaldehyde の定量は Stotz の方法<sup>40)</sup>によつた。

### 3. Xanthine oxidase 活性測定

Dhungot の法<sup>41)</sup>により, 肝 Xanthine oxidase 活性を測定した。

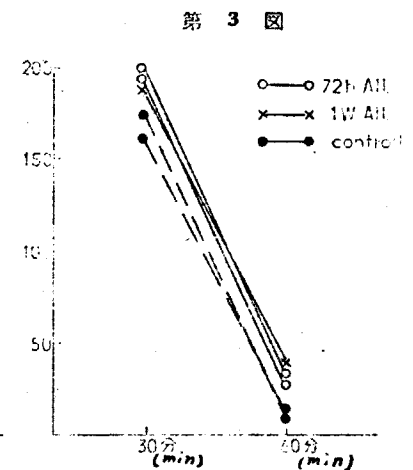
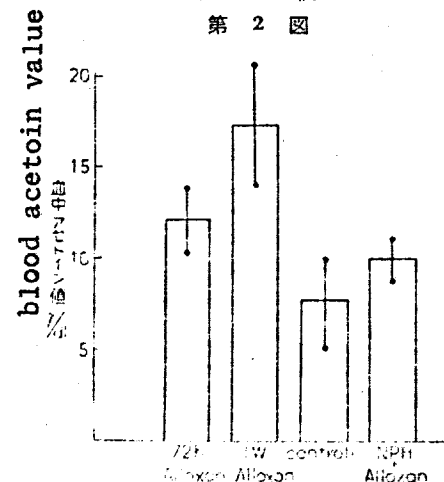
## 実験方法

約180 g 内外の標準 Wistar 系白ネズミを用い, 数日間雑食 (ひまわり玉蜀黍, 餅米, パン粉, 魚粉) と野菜で飼育した。Alloxan 白ネズミは alloxan を 200mg per kg 腹腔内に注射し, 72h, 1週間後に, 実験がマステールにて以上のものだけを用いた。B<sub>1</sub>欠乏群は本教室の基本合成食より B<sub>1</sub>を除去したもの, 又对照群等は基本合成食にて飼育した。又体重調節群は, 飼料をへらして, 体重を欠乏群と等しくなるように注意した。以上各群の白ネズミについて, 無処置, 並びに acetaldehyde 負荷時の血中, 肝, 腎, 尿中 acetoin 値, 又 acetaldehyde 負荷時の血中 acetaldehyde を測定し, 又肝 Xanthine oxidase 活性値をも測定した。以上の実験はすべて, 空腹1時間後に施行した。健康ネズミには無処置患者は前日より注射, 服薬を禁じ, 肝腎空腹時に肝腎臓より採血した後に血中 acetoin 値を定量した。

## III. 実験成績

### 1. Alloxan 白ネズミの血中 acetoin

Alloxan 白ネズミの血中 acetoin 値



Alloxan 注4, 1週間後の白ネズミ、および対照の各群6匹について、腹部大動脈から10ccずつ採血し測定に供した。その成績は第2表に示すごとく alloxan 白ネズミの acetoin 値は明らかに高値を示す。又 alloxan 注射24h後に全量を確定し、NPH insulin にて治療するに (0.1 unit per 100g 7日間連日腹注) acetoin 値は回復の傾向を示す。

#### Acetaldehyde 負荷時の血中 acetoin 値の変動

前記各群の白ネズミに acetaldehyde を15mg per 100g 腹注し、4匹ずつより5ccを30分、60分後に腹部大動脈より採血し測定した。その結果は第3表に示すごとく、両群共に従来いわれているように、著明な acetoin 値の増加が見られるが、alloxan 白ネズミの方が対照より増加度が著しい。

#### 2. Alloxan 白ネズミの肝腎 acetoin

Acetoin 代謝に於て、肝の占める位置はきわめて重要で、すでに Westerfeld 等は肝切除により血中 acetoin 値の増加する事を認めた<sup>1)</sup>。又生田等は血中 acetoin を負荷して、臓器別にその値を測定して、他の臓器では一様に増加しているにも関わらず、肝に於て特に低くなっている事より、肝で acetoin が代謝されるのではないかと考え、肝 homogenate と acetoin とを in cubation し acetoin の減量を報告した<sup>4)</sup>。一方腎臓の除去では血中 acetoin 値はほとんど変化しない<sup>2)</sup>。

#### Alloxan 白ネズミの肝 acetoin 値

Alloxan 白ネズミ及び対照を断頭瀕血し、肝3gに蒸留水3ccを加え、homogenate とし、その4ccを測定に供した。これを肝4gあたりの acetoin 値としてあらわすと、第1表のごとく両者に差はみられない。

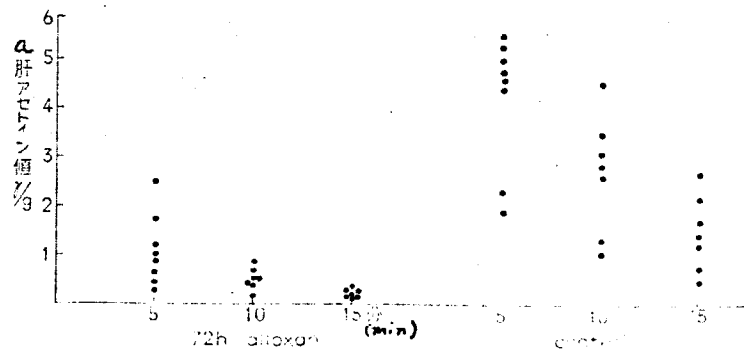
#### Acetaldehyde 負荷時の肝 acetoin 値

Acetaldehyde を45mg per 100g 腹腔内に注射して、5、10、15分後に断頭瀕血し肝 acetoin 値を測定した。両群共に増加するが、第4表に示すごとく、alloxan 白ネズミの方が明らかに低値を示す。

第1表

a 例数	b 体重	c 種類	d 肝 acetoin 値 g/4g	e 平均値
7	90	72h Alloxan	0.45, 0.39, 0.32, 0.22, 0.12, 0.30, 0.34	0.30
7	92	control	0.44, 0.38, 0.32, 0.28, 0.44, 0.12, 0.30	0.33

第4表



#### Alloxan 白ネズミの腎 acetoin 値

両群を断頭瀕血し、腎臓をとりだし、その1gと蒸留水3ccで homogenate をし、paste 3ccを測定に供した。これを腎4g中の acetoin 値としてあらわすと、第2表のごとく、両群間に差はみられない。

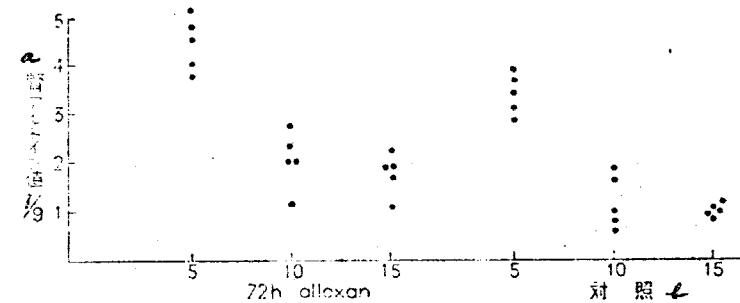
第2表

例数	体重	種類	腎 acetoin 値 g/4g	平均値
7	90	72h alloxan	0.95, 0.92, 0.80, 1.20, 1.0, 0.99	0.98
7	92	control	0.82, 1.8, 0.12, 0.95, 0.8, 1.0	0.91

#### Acetaldehyde 負荷時の腎 acetoin 値

前記各群に acetaldehyde を負荷し、5、10、15分後に腎 acetoin 値を測定すると、alloxan 白ネズミに於て、明かに第5表に示すごとく高値を示した。

第5表

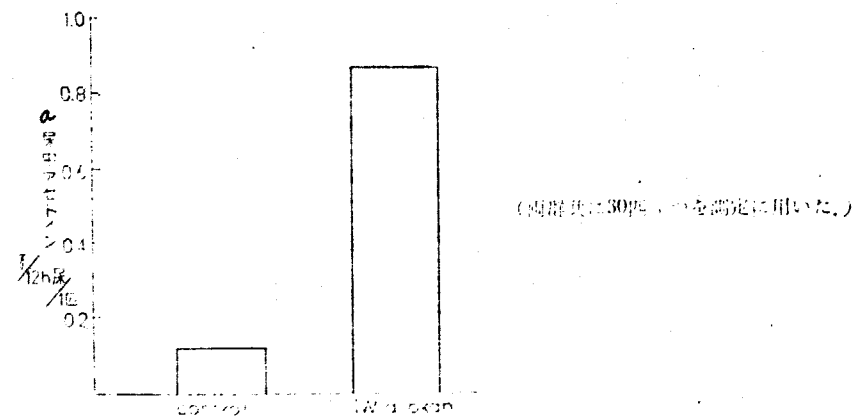


#### 3. Alloxan 白ネズミの尿中 acetoin

人尿中の acetoin についてはすでに多くの報告がある<sup>1)</sup>。宮地等は健康人に1NAH1日0.6gを5日間投与するに尿中 diacetyl は増加したが、これは酢酸 Ca を1日10mg投与、5日間続けたところ、かなりの減少を示したと云う。私共は両群の白ネズミを金おみに入れ、飼料を与えずに、水のみを与え、12時間尿を集め、尿中 acetoin を測定した。

第6表に示すごとく、alloxan 白ネズミは尿中 acetoin が明らかに高い。

第6表



#### 4. Alloxan 白ネズミに acetaldehyde 負荷時の血中 acetaldehyde

Alloxan 白ネズミ及び対照の各群に acetaldehyde を45mg per 100g 腹注し、60分後に腹部大動脈より1



採血 (側) 是也。其結果は第3表に示す如く, alloxan 白ネズミの血中には対照動物血中には acetaldehyde の停滞による結果を示す。

### 第3表

5. alloxan 白ネズミの肝 Xanthine oxidase 活性値  
堀田等は alloxan 糖尿病白ネズミの肝臓 B<sub>2</sub> の減少<sup>(46)</sup>,  
~~amino acid oxidase 活性値の低下<sup>(47)</sup>を主因として~~

amino acid oxidase 活性値の低下<sup>(47)</sup>, Xanthine oxidase 活性値の低下を報告した<sup>(48)</sup>。

alloxan 白ネズミに於ける acetaldehyde 分解の遅延  
Xanthine oxidase 活性値の低下を主因として報告した<sup>(49)</sup>。  
その肝 Xanthine oxidase 活性値を Axelord<sup>(50)</sup>  
Dhungat<sup>(51)</sup> 等は測定した。その肝 1g あたり 2 時間の  
Bul<sup>(52)</sup> と比較すると第7図に示す如く alloxan 白ネズミの肝  
対照より低下している。

### 6. 糖尿病患者の血中 acetoin

生野等は子宮がん患者血中 acetoin 値の増大<sup>(53)</sup>,  
又 Dawson 等は 50 以上の糖尿病患者血中の acetoin 値を測定  
した。その結果は増大を示す報告<sup>(54)</sup>。

和共は糖尿病患者、並に健康人の血中 acetoin 値  
を測定し、第4表に示す如く、糖尿病患者では高値を  
示すことが認められた。

### 第4表

27.8	9.8	22	13.5	15	9.4	30	12, 9,	7.5	11	24
4.5	9.1	8.2	6.0	7	10.5	4.8	5.4	3.8	9.4	0.9

# IV 考察

Acetoin, diacetyl 等の糖の代謝経路は尚未知の点が多い。最近 Juni 等は鶏胸筋を用いて、糖の代謝と diacetyl acetoin 等の糖の相互転換について、新しいデータを発表した。その大略を下記に示す。

一方 Fritz 等は mouse Ehrlich Ascites cell の homogenate 中で pyruvate から acetoin が生成することを示した。<sup>55)</sup> Roselwin 等は  $C^{14}$  labelled acetoin を用いて、 $C^{14}$  riboflavin (vit<sub>B2</sub>) の ring A 部分に  $C^{14}$  が取り込まれることを eremothecium ashybyae を用いて証明した。<sup>56)</sup>

Martin 等は B<sub>12</sub> 欠乏白ネズミに acetaldehyde を投与し、血中の acetaldehyde を定量すると、投与量は血中の acetaldehyde が消失する速度に比例し、更に acetoin 負荷による血中の acetoin 濃度も増加する。B<sub>12</sub> 欠乏白ネズミの血中の acetoin 濃度は正常と報告した。<sup>57)</sup>

Stotz 等は rat brain の homogenate 中で pyruvate と acetaldehyde とを 10 分間 incubate すると、acetoin が生成することを示した。B<sub>12</sub> 欠乏 rat brain の場合は生成が著しく速いことを示した。<sup>58)</sup> また、B<sub>12</sub> は carboxylase

第 8 回

The diagram illustrates the glyoxylate shunt pathway, which bypasses the decarboxylation steps of the TCA cycle. The pathway is as follows:

- Isocitrate** is converted to **α-ketoglutarate** and **Succinyl-CoA** by the enzyme **isocitrate lyase**.
- Succinyl-CoA** is converted to **Succinate** by the enzyme **succinyl-CoA lyase**.
- Succinate** is converted to **Malate** by the enzyme **succinate dehydrogenase**.
- Malate** is converted to **Oxalacetate** by the enzyme **malate dehydrogenase**.
- Oxalacetate** combines with **Acetyl-CoA** to form **Citrate**.
- Citrate** is converted to **Isocitrate** by the enzyme **citrate isomerase**.
- Isocitrate** is converted to **α-ketoglutarate** and **Succinyl-CoA** by the enzyme **isocitrate lyase**, completing the cycle.

The diagram also shows the conversion of **Succinyl-CoA** to **Succinate**, **Malate**, and **Oxalacetate**, and the conversion of **Oxalacetate** to **Citrate**.

第 5 表

第 5 表			
a	b	c	d
例	重	種	血中 acetoid %/dl
7	33	♀ B <sub>1</sub> 欠乏群	14.2
7	143	f 对照 群	6.8
7	90 g	♀ 体重抑制群	7.0

山形等は短核患者の血中 acetoin 値を測定し、正常に比しやや多量であつたが、その際胸気症状の有、無では明はみられなかつたと述べている<sup>92)</sup>。

宮地等は mous をパントロン酸欠乏にて飼育したところ、体内の diacetyl が正常の 3~4 倍に増加したと報告し、この事實は、diacetyl が acetyl CoA となる際にパントロン酸欠乏により障害されたためであると述べている<sup>93)</sup>。

例 数	体 重	種 類	血中 acetoid $\gamma$ /dl
7	83	♀ B <sub>1</sub> 欠乏群	14.2
7	148	♀ 対 照 群	6.8
7	90 g	♀ 体重抑制群	7.0

従来より acetoin の生成には CoA は必要ないとされているが<sup>12)</sup>、Berry 等は rat brain のミトコンドリア Powder を用いた条件下で CoA は必要であると報告した<sup>13)</sup>。

さて alloxan 自体は特に害ける, CoA, 總 B<sub>12</sub>, lipoic acid の減少, acetyl 化能の低下は以上の事実よりいずとも, acetoin 値の増加に好適な場を提供している。

Acetaldehyde (負荷時の増加) acetaldehyde の分解は alloxan 自身によって行われ、肝 xanthine oxidase 活性の低下に起因すると思われる。

Acetaldehyde 負荷時の呼気acetoin 値がalloxan 負荷により一時的に上昇する意義は尚説明困難で、今後更に検討の要がある。

三、

$a$	$b$	$c$	血中 acetoin 值 r/dl	$d$ 平均
13 <sup>e</sup>	9.8	22	13.5 15 9.4 30 12, 9, 7.5 11	24 15.8
10 <sup>f</sup>			6.0 7 10.5 4.8 5.4 3.8 9.4	6.9

附

Aspirin, Heparin, Vitamin E, and other drugs may also be used to help prevent blood clots.

To decrease your risk of blood clots, you should:

- Take aspirin or another blood thinner as directed by your doctor.
- Stay active and move around often, even if you are sitting or lying down.
- Drink plenty of fluids, especially water.
- Avoid long periods of immobility, such as long flights or car trips.

If you have a blood clot, it is important to take medication as prescribed by your doctor. Stopping the medication suddenly can increase your risk of complications.

There are many ways to prevent blood clots. Talk to your doctor about the best way to protect yourself.

Source: American Heart Association

[illegible][illegible]

the 1990s, the number of people in the world who are under 15 years of age is expected to increase from 1.1 billion to 1.4 billion. The number of people aged 65 and over is expected to increase from 200 million to 400 million. The number of people aged 15 and over is expected to increase from 3.5 billion to 4.5 billion. The number of people aged 15 and over is expected to increase from 3.5 billion to 4.5 billion. The number of people aged 15 and over is expected to increase from 3.5 billion to 4.5 billion.

## V 結 論

- 1) Alloxan 負荷による血中 acetoin 値は対照より高値を示した。
- 2) Alloxan 負荷による acetaldehyde を負荷し、血中 acetaldehyde, acetoin を測るといざれば対照より高値を示すが、血 acetoin 値は逆に低値を示す。
- 3) 糖尿病性患者血中 acetoin 値は健康より高値を示すものが多い。

(終りに臨み、無常の世に別れ、二山間弘二居士に御礼申上りて上り)

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## Quality of Commercial Buttermilks<sup>1</sup>

J. Dairy Sci. 51(2):226--  
227

### Abstract

Buttermilks from ten regional dairies were judged for flavor and analyzed for acetaldehyde, diacetyl, volatile acidity, and microbial quality. Large variations in flavor score and in concentration of acetaldehyde, diacetyl, and volatile acidity were observed. In none of the samples was the diacetyl:acetaldehyde ratio near the 4:1 value reported to be necessary for a good flavor balance. In contrast to the objective chemical data, the microbial quality of these buttermilks was good.

In recent years, much progress has been made in the chemical definition of the characteristic flavor of cultured buttermilk. It is now accepted that lactic acid, diacetyl, acetaldehyde, dimethyl sulfide, and acetic acid are the most important flavor compounds in buttermilk (4), and the quantitative relationships of these compounds are important in defining buttermilk flavor. Nevertheless, the development of sufficient amounts of desirable culture flavor and aroma is still one of the major problems confronting the cultured dairy products industry.

A number of methods for providing enhanced culture flavor have been proposed. These include the use of modified cultures, starter distillates, and direct addition of diacetyl to the product. These methods improve flavor, but the attainment of uniform, desirable culture flavor is not easily achieved (4). Recently, Lindsay et al. (4) described the formulation of butter culture flavor concentrates. Their results indicated that a large preference panel showed equal preference for high quality naturally cultured products and artificially flavored products. The present study was made to show the variation in commercially available buttermilk and provide additional support for the use of synthetic flavor concentrates in the manufacture of this product.

### Experimental Procedure

Buttermilk samples from ten different re-

<sup>1</sup>Supported in part by funds granted by the American Dairy Association.

gional dairies were obtained from commercial outlets. These samples were coded and scored for flavor by four experienced judges. A scoring range of 30 to 40 was used, 40 being assigned as a standard when there was no flavor criticism. Diacetyl and acetaldehyde contents of samples were quantified in duplicate by previously reported colorimetric methods (2, 6). To determine total volatile acidity, 100 g of each sample was acidified to pH 4 with concentrated phosphoric acid and steam distilled until 100 ml of distillate was collected. Distillates were titrated to a phenolphthalein end point with 0.10 N sodium hydroxide. Results are expressed as milliliters of base to reach the end point (7).

The samples were checked for coliforms by plating aliquots in Desoxycholate Agar (Difco) and incubating at 37 C for 24 hr. As an indication of the number of spoilage organisms present, CVT counts were made by plating samples in standard plate count agar (Difco) containing crystal violet and 2,3,5-triphenyltetrazolium chloride as described by Olson (5).

### Results and Discussion

Results of flavor, chemical, and microbial analyses of the ten buttermilk samples are shown in Table 1. Rather large variations in concentrations of diacetyl and volatile acidity and pH values are apparent. Flavor scores ranged from 34 to 39, and the judges were in agreement that most of the samples lacked a well-balanced culture flavor. The ratio of diacetyl to acetaldehyde in the samples showed considerable variation. According to Lindsay et al. (3), while it is the absolute quantities of these two compounds that determine the flavor intensity, the relative amounts control flavor balance. It is noteworthy that in two of the samples was the diacetyl-to-acetaldehyde ratio near the 4:1 value reported by Lindsay et al. (3) to be necessary for a good flavor balance in butter cultures. Further, many samples exhibited off-flavors such as cooked or stale.

Volatile acidity values for these samples were within a range of about 5 to 7.5 ml. In view of the recent report that volatile acidity is

TABLE 1  
Chemical, microbial, and flavor data for buttermilks analyzed

pH	Titrateable acidity <sup>a</sup>	Diacetyl (ppm)	Acetaldehyde (ppm)	Diacetyl: acetaldehyde ratio	Volatile acidity <sup>b</sup>	Flavor score	Coliforms	CVT count
4.32	0.81	1.68	0.05	33.6:1	7.54	38	0	0
4.29	0.84	3.55	0.18	19.7:1	6.32	39	0	0
4.31	0.80	2.78	0.27	10.3:1	6.31	36.5	0	1
4.32	0.82	1.86	0.15	12.4:1	6.23	37	0	4
4.52	0.78	0.77	0.06	12.8:1	6.04	34	0	2
4.41	0.79	1.12	0.13	8.6:1	4.98	36	0	14
4.42	0.79	3.00	0.16	18.8:1	5.97	36.5	0	1
4.56	0.78	1.90	0.16	11.9:1	6.66	37.5	0	220
4.42	0.80	0.00	0.00	.....	.....	36	0	0
4.45	0.82	0.41	0.36	1.1:1	5.54	35	0	0

<sup>a</sup> Expressed as per cent lactic acid.  
<sup>b</sup> Expressed as ml of 0.10 N base.

A indicator of flavor in sour cream, this amount of variation may be significant (1). There was no apparent relationship between pH or titratable acidity and volatile acidity. In contrast to the wide variations in objective chemical data, the microbial quality of the buttermilks was good. All samples were norm-negative. The CVT counts were low except for Sample H. These counts are indicative of post-pasteurization contamination. Although a limited number of samples were tested in this study, the wide variation in levels of key flavor compounds noted suggests that consistency in flavor production is still difficult to attain using natural culturing processes in commercial operations. In the manufacture of nearly all major food items, the quality of flavor is widely sought. The available knowledge on the use of synthetic culture concentrates should make it possible for the dairy industry to provide the consumer with products having a well-balanced, consistent flavor. From our data, this is obviously not yet accomplished.

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February 28, 1936

## MEMORANDUM FOR DR. ELLIOTT:

The questions raised in the letter of February 15 from the Head of the Department of Dairy Husbandry, College of Agriculture, Agricultural Experiment Station, Urbana, Illinois, necessitate consideration of the whole question of added flavor in butter.

Researches have shown that the flavor in butter is largely due to diacetyl. This substance, along with its precursor acetyl methyl carbinol, is formed by action of bacteria normal to milk on milk constituents, and therefore both of these substances are present in butter made by the general practice, dating back to primitive times, of using sour milk or cream for butter making. The history of butter starters, reported in Food Control Statement No. 37, p. 10, shows that intentional souring of milk and cream for butter making, by inoculation with selected sour milk, predates the science of bacteriology. Present-day starters are a result of the development of biological control methods, so that now they generally involve the use of definitely chosen and controlled bacterial strains. They are used in two ways, either to ripen the whole batch of cream before churning or to ripen a small batch, which is added to the total during churning. Their use in either way was a well established practice in 1923, when the Federal standard for butter became effective, defining butter as the "product usually known as butter". By this definition the use of starters would appear to have been established as part of the butter making process.

Butter flavors of the diacetyl type are prepared, we understand, by biological action of bacteria of the starter type on milk, with subsequent separation and concentration of the end products. The proposal to steam distill starter and add the distillate to the butter is intermediate between the use of starter and the use of diacetyl flavor. The steam distillate of the starter would contain diacetyl in an unpurified, diluted condition. Recognition of the proposed practice would be an entering wedge toward recognition of the use of commercial flavors. Although the use of starters, because of established custom in butter making, enters into the understanding of the product usually known as butter, the manipulation of the starters and addition of distillate is not a step in normal butter production. This is a deviation from general trade practice and consumer understanding and knowledge. Because of almost universal butter production on the farm, the large proportion of the population knows how butter is made. In deciding whether the practice is to be recognized as proper we are confronted with the decision of the Supreme Court in the Douglas Vinegar case that processes employed are not material in the test of identity. Just how far we can go to maintain that cream treated with distillate from starter and churned does not produce the identical article butter is not readily determinable. It is not strictly the addition of the same constituent although it is doubtful if the difference could be analytically demonstrated. Having held that the addition of butter flavor (diacetyl) is adulteration, we should likewise hold that the proposed practice is not appropriate in butter production. In recommending this position it is realized that it will perhaps be difficult to convince a court and jury of the logic of the fine distinction we have drawn.

H. A. LEPPER

H. A. Lepper

HAL/avr

C.R.S.B. 122:373-374, 1936

## Antiseptic and Bactericidal Action of Diacetyl

on Various Pathogenic Bacteria

by M. Levy-Bruhl and Yvonne Cado

The products of the butylene-glucolic fermentation: 2-3-butylene-glycol, acetylmethylcarbinol and diacetyl are constant factors of the metabolism of living organisms. [M. Lemoigne (1)].

Experiments done to study the usage of diacetyl showed that in doses of from 0.4 g to 0.8 g per liter, it is toxic for a certain number of bacteria (1), a fact recently confirmed for various lactic and saprophytic leavening agents. [M. Lemoigne and P. Monguillon (2)].

	Quantity of diacetyl per 1000 cc of milieu, in g.						Control tube without diacetyl
	4	2	1	0.4	0.2	0.1	
Staphylococcus aureus ....	0	0	0	++	+++	+++	+++
Streptococcus .....	0	0	0	0	+	++	+++
Pneumococcus .....	0	0	0	0	++	+++	+++
Gonococcus .....	0	0	0	0	0	+	+++
P. pyocyanea .....	0	0	0	0	+++	+++	+++
					(in 5d)		
Pneumobacillus .....	0	0	0	0	++	+++	+++
					(in 5d)		
Colibacillus .....	0	0	0	0	++	+++	+++
					(in 5d)		
Salmonella typhosa .....	0	0	0	0	+	++	+++
Salmonella paratyphi A ...	0	0	0	0	++	+++	+++
Salmonella paratyphi B ...	0	0	0	0	++	+++	+++
					(agglutinated sample)		
Corynebacterium diphtheriae in ordinary milieu .....	0	0	0	0	0	+++	+++
					(in 4d)		
Corynebacterium diphtheriae in horse serum .....	0	0	0	0	+	+++	+++

The results, without special comment, were recorded after 48 hours.  
 + = small amount of culture, ++ = medium amount of culture,  
 +++ = large amount of culture.



Following the suggestions of M. Lemoigne, it seemed interesting to us to study whether similar properties manifested themselves in regard to certain pathogenic bacteria. First of all, we studied what amounts of diacetyl prevent bacterial development by adding increasing quantities to the milieux of usual culture liquids: a bean broth, peptonized and sweetened for most bacteria, an ascitic liquid for gonococcus, horse serum for diphtheria bacilli. We made sure that this addition did not significantly change the pH of the milieux.

The results obtained are summarized in the table.

We thus found that a proportion of 0.4 g per 1000 is sufficient to prevent almost all bacterial cultures.

Also, we tried to find what amounts would be bactericidal to certain of these microorganisms; in other words, the proportionate amount of diacetyl capable of killing bacteria in liquid cultures. For streptococcus, pneumobacillus, colibacillus, salmonella typhosa and salmonella paratyphi A, an amount of 0.4 g of diacetyl per 1000 was shown to be fatal.

It can be seen that this amount of diacetyl corresponds to the smallest preventive dose. We propose to study whether lesser amounts will still be toxic for the same bacteria.

The fact that diacetyl is not eliminated and is actually used and transformed by higher organisms may constitute a favorable factor for its therapeutic use as a bactericidal agent. Its bactericidal effect is quite inferior to that of certain mineral substances (mercury chloride) or dyes (gonacrine), and is about the same as that of oxyquinoline and some of its derivatives whose practical usage has brought some interesting results.

- (1) M. Lemoigne, C.R. de la Soc. de Biol., 1927, 97, p. 1479
- (2) M. Lemoigne and P. Monguillon, Milk, 1936, 16, p. 26

Compt. rend. Soc. biol. 122: 373-4, (1936)

POUVOIR ANTISEPTIQUE ET BACTÉRICIDE  
DU DIACÉTYLE VIS-À-VIS DE QUELQUES MICROBES PATHOGÈNES,

par M. LÉVY-BRUHL et YVONNE CABO.

Les produits de la fermentation butylique-glycolique (1-3 butylique-glycol, acétyl-méthylcarbinol et diacétyl) sont des termes constants du métabolisme des êtres vivants [M. Lemoigne (1\*)].

Des essais effectués pour étudier l'utilisation du diacétyl ont montré qu'à la dose de 0,4 gr. à 0,8 gr. par litre, il est toxique pour un certain nombre de microbes (1), fait confirmé récemment pour les ferments lactiques et saprophytes divers [M. Lemoigne et P. Monguillon (2\*)].

	Quantité de diacétyl p. 1.000 c.c. de milieu, en gr.						Tube témoin sans diacétyl
	4	2	1	0,4	0,2	0,1	
Staphylocoque doré .....	0	0	0	+	++	+++	+++
Streptocoque .....	0	0	0	0	+	++	+++
Pneumocoque .....	0	0	0	0	++	+++	+++
Gonocoque .....	0	0	0	0	0	+	+++
Pyocyanique .....	0	0	0	0	+++	+++	+++
					en 5 j.		
Pneumobacille .....	0	0	0	0	+	+++	+++
					en 5 j.		
Colibacille .....	0	0	0	0	+	+++	+++
					en 5 j.		
Bacille typhique .....	0	0	0	0	+	++	+++
Bacille paratyphique A ..	0	0	0	0	++	+++	+++
Bacille paratyphique B ..	0	0	0	0	++	+++	+++
					pousse agglutiné		
Bacille diphtérique en mi- lieu ordinaire .....	0	0	0	0	0	+++	+++
					en 4 j.		
Bacille diphtérique sur sé- rum de cheval .....	0	0	0	0	+	+++	+++

Les résultats, sauf indication spéciale, ont été notés au bout de 48 heures.  
+ = culture peu abondante, ++ = abondance moyenne, +++ = culture riche.

Suivant les suggestions de M. Lemoigne, il nous a paru intéressant de rechercher si des propriétés analogues se manifestent à l'égard de certaines bactéries pathogènes. Nous avons étudié tout d'abord les doses de diacétyl empêchant le développement microbien par l'addition de quantités croissantes, à des milieux de culture liquides usuels : bouillon de haricots peptoné glucosé pour la plupart des bactéries, bouillon ascite pour le gonocoque,

sérum de cheval pour le bacille diphtérique. Cette addition, comme nous nous en sommes assurés, ne modifie pas sensiblement le pH du milieu.

Les résultats obtenus sont résumés dans le tableau ci-joint.

Nous constatons donc qu'une proportion de 0,4 gr. p. 1.000 suffit à empêcher presque toute culture microbienne.

Nous avons recherché d'autre part les doses bactéricides vis-à-vis de certains de ces microorganismes; c'est-à-dire les proportions de diacétyl capables de tuer ces microbes en cultures liquides. Pour le streptocoque, le pneumobacille, le colibacille, le bacille typhique et le paratyphique A une dose de 0,4 gr. de diacétyl p. 1.000 s'est montrée mortelle.

Cette proportion de diacétyl correspond, comme on le voit, à la plus petite dose empêchante. Nous nous proposons de rechercher si des doses inférieures, peuvent encore se montrer toxiques pour ces mêmes bactéries.

Le fait que le diacétyl n'est pas éliminé et se trouve vraisemblablement utilisé et transformé par les organismes supérieurs pourrait constituer un élément favorable pour une application thérapeutique de cette action bactéricide. Celle-ci, très inférieure certes à celle de certaines substances minérales (chlorure de mercure) ou colorantes (gonacrine), est du même ordre que celle de l'oxyquinoléine et de certains de ses dérivés dont l'utilisation pratique a donné des résultats intéressants.

(Institut Pasteur.)

(1\*) M. Lemoigne, C. R. de la Soc. de biol., 107, 1, 1924, p. 1479.

(2\*) M. Lemoigne et P. Monguillon, La Tait., 1936, t. 10, p. 36.

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# Symposium on Foods: The Chemistry and Physiology of Flavors

*The fourth in a series of symposia on  
foods held at Oregon State University*

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R. C. Lindsay

Cultured Dairy Products

## INTRODUCTION

Lactic acid-producing bacteria have been used for centuries to convert fresh milk into edible fermented dairy products. Before the advent of pasteurization, refrigeration, and refined culturing procedures, man relied on the natural microbial flora or crude starters to carry out the fermentation processes. Through the years a great number of cheeses and fermented milk and cream products have evolved. This paper deals with a group of fresh dairy products which possess a common culture flavor. Typical examples of this group are butter culture, cultured buttermilk, cultured cream butter, sour cream, and creamed cottage cheese. The characteristic culture flavor of these products is primarily dependent upon flavor and aroma substances produced by certain lactic acid-producing bacteria.

## FLAVOR CONTRIBUTIONS FROM NORMAL MILK CONSTITUENTS

Although culture flavor is dependent on microbial metabolism, it is generally recognized that a significant contribution to the overall flavor of cultured products is made by normal milk constituents. The compounds considered hereunder are naturally present in fresh milk or are produced from milk constituents during manufacture.

Milkfat possesses several characteristics which are believed to contribute to the flavor of cultured products. The glycerides of milkfat contain considerable quantities of butyric, caproic, caprylic, and capric acids. Work by Harper *et al.* (1961) and Khatri and Day (1962) has demonstrated that milk contains appreciable levels of unmethylated fatty acids (straight chain  $C_{18}$  or less). At optimum concentrations these fatty acids are believed to add favorable notes to the flavor of dairy products. A number of less abundant fatty acids have also been found in the lipids of cows' milk (Magidman *et al.* 1962). The flavor significance of the majority of these acids is largely unknown. Of particular interest are several isomeric unsaturated 18 carbon atom fatty acids isolated from milkfat by de Jong and van der Wal (1964). These acids are believed to be precursors of 4-*cis*-heptenal, a compound isolated from milkfat which exhibits a cream flavor (Begemann and Koster 1964). The cream flavored component is formed through the autoxidation of the isolinoleic acids.

Work by Boldingh and Taylor (1962) on the trace constituents of milkfat has demonstrated the presence of several compounds which probably are of significance in butter flavor. These workers have isolated and identified several optically active lactones from milkfat. The delta-lactones with 8, 10, 12, 14, and 16 carbon atoms were found to comprise the major portion of the lactone fraction. Indirect evidence indicated that hydroxy-acids were esterified in normal glycerides. Hydrolysis of the hydroxy-acids from glycerides and subsequent ring closure would give rise to the corresponding lactones.

When milkfat is heated in the presence of water, an homologous series of methyl ketones with odd-numbers of carbon atoms is produced (Patton and Tharp 1959). The methyl ketones are formed through the decarboxylation of even-numbered beta-ketoacids which are normally esterified to triglycerides of milkfat (Parks *et al.* 1964). It is generally accepted that methyl ketones contribute to the flavor of cultured products, and especially those containing significant quantities of milkfat. Recent work has indicated that butter cultures prepared from heated whole milk contained larger quantities of 2-pentanone and 2-heptanone than did control heated milk (Lindsay *et al.* 1965B). Since the beta-ketoacid decarboxylation reaction is pH dependent (Widmark 1920), it has been postulated that the lower pH of butter culture causes a more rapid formation of methyl ketones.

Patton and co-workers (1956) first isolated dimethyl sulfide from milk and found that at its flavor threshold (12 p.p.b. in water) it exhibited a typical milk odor. Day *et al.* (1964) have found dimethyl sulfide in butter cultures, cultured cream butter, and sweet cream butter. It was noted that dimethyl sulfide was a significant flavor component in fresh sweet cream butter, and that it had the capacity to smooth out the harsh flavor of diacetyl and acids associated with culture flavor.

All cultured dairy products receive some degree of heat treatment during the manufacturing processes. A variety of flavors develop when milk is heated. The severity of heat treatment determines the type and intensity of flavor that will be apparent. The flavor significance of compounds, such as 2-furfural, 2-furfuryl alcohol, maltol, and formic acid which are formed in milk by various degrees of heat treatment, is not yet clear. Undoubtedly, these compounds and other thermally induced compounds contribute to the flavor of cultured products which receive substantial heat treatment.

The term "butter flavor" has been used synonymously with culture or cultured cream butter flavor. At the present time, the assumption that the two flavors are the same is not entirely correct. In recent years the majority of high quality butter manufactured in the United States has

been made from pasteurized sweet cream. While sweet cream butter possesses the unique and desirable characteristics peculiar to milkfat, it has a bland flavor and does not possess a culture flavor. The flavor of sweet cream butter, therefore, is due entirely to flavor compounds initially present in fresh cream and to those produced or added (for example, salt) during manufacture. As yet, it has not been possible to duplicate synthetically the natural flavor of sweet cream butter.

On the other hand, cultured cream butter was the usual product before the development of modern processing procedures. In many instances culture flavor production was brought about by the natural bacterial flora of the cream rather than sophisticated culturing techniques. Clearly, cultured cream butter flavor is due to both inherent natural flavor compounds and compounds produced by microbial activity. Therefore, it is proposed that the terms, "cultured butter flavor" or "cultured cream butter flavor," be used in describing the flavor of butter processing a true culture flavor. The term, "butter flavor," would then be a more meaningful description of the flavor of sweet cream butter.

#### ORGANISMS PRODUCING CULTURE FLAVOR

The bacteria found in starters used for the manufacture of cultured products can be placed into three general categories. These are (1) the lactic acid-producing streptococci, *Streptococcus lactis* and *Streptococcus cremoris*; (2) the associative citrate-fermenting aroma bacteria, *Leuconostoc citrovorum* and *Leuconostoc dextranicum*; and (3) the dual purpose lactic acid- and aroma-producing strains of *Streptococcus diacetylactis*. For convenience the term, "aroma bacteria," is used as a general term to describe those organisms which produce appreciable quantities of diacetyl when appropriately grown in a milk medium.

Commercially blended mixed-strain starter cultures contain combinations of the above mentioned species. The type of product that is to be manufactured determines the species of organisms to be included in the culture. For instance, in setting a vat of cottage cheese only lactic acid for coagulation of milk is necessary. In this case combinations of strains of *S. cremoris* and *S. lactis* may be quite satisfactory for the production of a bland flavored product. However, when culture flavor is desired some provision for the inclusion of aroma bacteria must be made. For creamed cottage cheese manufacture some culture flavor may be obtained by employing mixed-strain cultures containing aroma bacteria for cheese setting. Frequently aroma bacteria produce considerable quantities of carbon dioxide through citrate fermentation and this leads to problems with floating curd during the cooking process. Alternate natural

culturing methods for producing culture flavor have been developed. These methods involve adding cultures of *L. citrovorum* (Mather and Babel 1959) or *S. diacetylactis* (Lundstedt and Fogg 1962) to the creaming mixture prior to final mixing. These processes eliminate floating curd problems caused by the aroma bacteria. Carbon dioxide production in the finished cheese is not objectionable.

Starter cultures are routinely propagated in milk media. Culture activity is maintained by frequent transfers. Experience has shown, however, that it is very difficult to achieve consistency in flavor production under normal dairy plant operations. Many factors are involved in the difficulties encountered in the production of high-quality aroma producing cultures. Changes in the balance of the microbial flora are directly reflected in the flavor and acid producing characteristics. Certain culturing procedures tend to favor the development of some bacterial strains over others (Galesloot 1962; Vedamuthu *et al.* 1964). Quite often a shift in the balance of organisms is experienced, and one or more strains predominate after a few transfers. The usual result is the loss of the ability of the culture to produce a desirable culture flavor. The use of culture rotation programs and single-use starters which do not require routine dairy plant propagation has been of value in cultured product manufacture. However, the dairy industry still is faced with starter problems.

#### STARTER DISTILLATE AS A SOURCE OF CULTURE FLAVOR

→ Starter distillates have been used for producing culture flavor in butter, sour cream, and creamed cottage cheese. These flavor concentrates are obtained from desirable mixed-strain butter cultures that exhibit high flavor-producing characteristics. In addition, culturing methods, such as the addition of citric acid to the medium, are employed to enhance flavor production. Steam distillation is used to recover the volatile flavor fraction from ripened cultures. The flavor intensity of such distillates can be standardized to meet market demands.

Although a starter distillate imparts a definite culture flavor, it is well accepted that it does not duplicate the balanced flavor obtained through the use of natural culturing procedures. Several factors may contribute to the inability of starter distillates to produce exact flavor duplication. Loss of desirable volatile flavor compounds and production of heat-induced components during steam distillation may change the flavor properties of starter distillates. Furthermore, the ratio of desirable flavor compounds found in natural intact butter cultures may be altered during distillation.

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" Synthetic Culture Flavor

Diacetyl has been used for imparting flavor to butter (Riel and Gibson 1961) but the results were not satisfactory. Work by Andersen (1961) indicated that addition of diacetyl to single strain cultures of *S. lactis* and *S. cremoris* ~~xxxx~~ resulted in an improved flavor...In most instances starter distillate gave a more desirable flavor than the synthetic formulations. This emphasizes the fact that even though the flavoring properties of starter distillates are inferior to natural culturing procedures, the distillates contain a reasonably complete spectrum of culture flavor compounds."

Nature 167:338-342, 1951  
QUALITATIVE ASPECTS OF THE  
CHEMISTRY AND BIOLOGY OF  
RADIOMIMETIC (MUTAGENIC)  
SUBSTANCES

By ANTHONY LOVELESS

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A RECENT communication<sup>1</sup> set forth the precise biological criteria upon which chemical substances may be classified as radiomimetic and thereby distinguished from other types of 'mitotic poisons'. Cytologically, the term 'radiomimetic' must be restricted to those substances which can be shown unequivocally to react upon the resting cell in such a way as to produce an alteration of the genetic material which is revealed by the appearance of chromosome breakage and rearrangement in subsequent divisions. It is becoming evident through work of geneticists, notably Auerbach and Robson<sup>2</sup>, Rapoport<sup>3,4</sup>, Demerec<sup>5</sup> and Bird<sup>6</sup>, that these substances are also mutagenic. Probably the converse is true, namely, that compounds which are radiomimetic as judged by genetical tests are also capable of producing visible chromosome effects in somatic tissues. Since, however, certain compounds have been claimed to be mutagenic without radiomimetic effects having been demonstrated cytologically, the word 'mutagenic' is enclosed in brackets in the title of this article.

Again, the terms 'radiomimetic' and 'mutagenic' have both qualitative and quantitative aspects. Thus, Auerbach<sup>2</sup> found that the proportion of translocations and sex-linked lethals produced in *Drosophila* by mustard gas was not the same as that produced by X-rays. Ford<sup>7</sup> observed that 'nitrogen mustard' (methyl-di-2-chloroethylamine) gave a different pattern of breakage in *HeLa* chromosomes to X-rays; Revell (ref. 1 and unpublished) has extended these findings to other substances of the radiomimetic class and endeavoured to relate high-breakage regions to the distribution of heterochromatin as defined by

low-temperature sensitivity. Accordingly, the results of our recent work will be reported in two parts. The present communication deals with the relation between chemical structure and radiomimetic activity. A later paper, written jointly with Mr. S. H. Revell, will describe the methods employed and the results obtained in a precise analysis of the cytological effects of active compounds in comparison with X-rays.

### Material and Methods

Throughout the majority of the work, root tip meristems of *Vicia Faba* have been used as a test object; a few experiments have also been done with larvae of *Tribolium palmarum*. It is necessary in an experiment of this kind that the duration of treatment be limited, so that immediate toxic effects and true genetic effects of the substance under test may be distinguished<sup>1</sup>. The assumption has therefore been made that the effect is determined quantitatively by the product of concentration and time. (It is appreciated that the concentration of reagent to which the material is exposed may not bear any direct relation to the concentration at the site of action in the cell. However, we are here dealing with compounds that act by covalent bond formation and not with indifferent substances of a narcotic type.)

Exposure of the roots to solutions of the compounds was, therefore, limited to one hour, and a wide series of concentrations was tested. Samples were taken at 4-6 hours after treatment for the observation of immediate effects on those cells already in division at the time of treatment, and at 18, 24, 36 or 48 hours as necessary to determine the effect upon cells in the resting stage at the time of treatment. Preparation of material for microscopic examination throughout the series of experiments reported here was by the Feulgen squash method.

### Choice of Compounds for Test

At first, attention was mainly focused upon compounds of the mustard type, since they are known to be radiomimetic and mutagenic and particularly, owing to the development of aliphatic- and aromatic-type nitrogen mustards by Goodman *et al.*<sup>12</sup> and Haddow, Kon and Ross<sup>13</sup> respectively, as growth inhibitors. Moreover, the chemical reactions of both sulphur and nitrogen mustards have been extensively studied, and it was known that these compounds react readily with the  $-NH_2$ ,  $-SH$ , carboxyl and imidazole groups of proteins and with amino- and primary and secondary phosphoryl groups of nucleic acids. The reactions of aromatic nitrogen mustards with amines, thiols and anions have recently been extensively studied by Ross and colleagues<sup>14-17</sup>.

In an attempt to decide which of these reactions were significant in determining the chromosome changes, a range of compounds was tested, designed so far as possible as selective reagents for the individual groups concerned. In the first instance, only bifunctional compounds were employed, but, where activity was found, monofunctional analogues were also tested. Sulphydryl reagents were the first selected in spite of the fact that the  $-SH$  reaction appears to play little part in the determination of other biological properties so far as sulphur mustard is concerned<sup>18</sup>. The choice was largely influenced by the belief held by many workers<sup>12-21</sup> that sulphydryl compounds play a vital part in cell division and growth and in the function of allegedly 'radiomimetic' poisons<sup>22,23</sup>. In addition, Ogston<sup>24</sup> has

demonstrated a very high 'competition factor' for mustard gas exerted by certain  $-SH$  compounds. Three main types of  $-SH$  reagent were employed, namely, iodoacetyl derivatives, organic arsenicals and unsaturated compounds of various double-bond characteristics. Some of these compounds may also react with amines, but in addition certain other amine reactants, notably carbonyl compounds, were tested. Thirdly, various reagents have been investigated which, like the mustards, are electrophilic, that is, they tend to react predominantly with free amino-groups and anions. Ross<sup>17</sup> has demonstrated that the groups most likely to react *in vivo*, assuming that the pH at different cell-sites does not vary appreciably from neutrality, are acid anions and certain aromatic-type amino-groups in nucleic acids.

The types of compounds chosen were largely based upon a list drawn up by Dr. W. C. J. Ross and myself more than two years ago as part of a programme designed to test the value of the cross-linkage hypothesis<sup>25</sup>. During the course of this work, the results of Ford and of Revell, already noted, became available, and an explanation was being sought for the apparent difference between the effect of X-rays, which was assumed to be mediated by a free-radical mechanism, and that of mustards and epoxides, which almost certainly act by alkylation or esterification. Hence, peroxides and per-salts which might act as chemical sources of hydroxyl radicals were also examined, especially since compounds of this type had been found by Dickey, Cleland and Lotz<sup>26</sup> to produce mutations in *Neurospora*. The results of these tests will be indicated briefly and dealt with more fully elsewhere in the later paper.

### Results

Biological effects may be classified as 'pycnotic' or 'radiomimetic' (mutagenic). The former include effects such as 'stickiness', anaphase lagging, 'sticky' bridge formation, and metaphase and telophase pycnosis, which are observed in cells dividing at the time of, or very shortly after, treatment.

It is true that intercalary stickiness at anaphase may occasionally result in fragment formation during anaphase (cf. Levan<sup>27</sup>, D'Amato<sup>28</sup>), and this may partly account for the low incidence of mutations after treatment with non-radiomimetic agents, for example, allyl isothiocyanate and dichloroacetone<sup>29</sup>.

### Bifunctional Compounds

**$-SH$  Reagents.** Those tested included the following: 1:2 di-(iodoacetyl-amino)benzene, 1:2 di-(iodoacetoxy)benzene, *syn* di-iodoacetone (analogous to dichloroacetone), *o*-xylol dibromide, 4-amino 3-hydroxy-phenyl arsenoxide,  $\beta$ -chlorovinylarsonic acid, lewisite, divinyl ether, diallyl sulphide, diallylamine, diallylaniline, methylallylamine, diallylamine methiodide, divinyl sulphone, dichlorodimethyl sulphone, protoanemonin, anemonin, patulin, hydroquinone, benzoquinone, allyl isothiocyanate. (The first substance, 1:2 di-(iodoacetyl-amino)benzene, has been reported as producing radiomimetic effects in *Triturus* (Colliero *et al.*<sup>30</sup>), but subsequent experiments have failed to repeat the result.) Of these, many gave pycnotic effects, but only protoanemonin, divinyl sulphone and, to a lesser extent, dichlorodimethyl sulphone were, in addition, radiomimetic. Unsaturated compounds in this list have been chosen to include examples with neutral groups (O), electron-repelling groups (N,S) and electron-attracting groups



( $\text{N}^+$ ,  $\text{SO}_2$ , lactone) adjacent to the double bond. Probably the first two types do not readily undergo addition reactions; in the third case, the double bonds are highly polarized and will react readily with nucleophilic groups.

— $\text{NH}_2$  reactants. The original list included certain amino-group reagents, such as ketenes and isocyanates, which it has not been considered profitable to pursue owing to the synthetic problems offered and the instability of such compounds in water. One acid chloride, *ortho*-phthalyl chloride, has been tested and found inactive, but subsequent examination by Dr. Ross (private communication) has revealed that this compound undergoes very rapid hydrolysis in water. The remainder which were tested are as follows: glyoxal, diacetyl, acetyl propionyl, phthalaldehyde, formaldehyde, methylal, resorcinol dichloroformate. None showed radiomimetic behaviour.

**Electrophilic reagents.** The nitrogen and sulphur mustards have been re-investigated for certain limited purposes: to determine the order of concentration required to give optimal effects in *Vicia*, and to investigate the possible influence of basic substituents. Diepoxides were prepared by Mr. J. L. Everett and Dr. W. C. J. Ross, of this Institute, as a development of the cross-linkage hypothesis (cf. Loveless and Revell<sup>1</sup>, Speakman<sup>2</sup>, and Deuel and Neukom<sup>21</sup>). In addition, a bi- and a tri-functional derivative of ethylenimine were examined.

All types proved to be effective radiomimetic agents and to show no non-specific toxic or pycnetic effects at active concentrations. Compounds tested were: mustard gas (dichlorodiethyl sulphide), nitrogen mustard (di-2-chloroethyl methylamine), dichlorodiethylamine, NN-di-2-chloroethyl-*p*-phenylene diamine, 1:2, 3:4-diepoxybutane, di(2:3-epoxypropyl)ether, 1:4 di-(2:3-epoxypropoxy)-benzene, diethylenimino phenyl methane, 2:4:6 triethylenimino-1:3:5 triazine.

### Monofunctional Compounds

Four types of bifunctional compounds have thus been found active, namely, sulphur and nitrogen mustards, diepoxides, ethylenimines and unsaturated compounds, in which a strongly electron-attracting group is immediately adjacent to the double bonds. Monofunctional analogues of these have therefore been investigated.

**Mustard series.** The first compound found active was a monofunctional analogue of mustard gas itself, ethyl-2-chloroethyl sulphide. A comparable 'one-armed' nitrogen mustard, dimethyl-2-chloroethylamine (analogous to methyl-*bis*-(2-chloroethyl)-amine), was afterwards found active when tested at comparatively high concentration and the solution made slightly alkaline to ensure that most of the compound was in the form of the free base.

**Epoxide series.** Low activity on the part of epichlorohydrin and butadiene-monoxide was discounted on the ground that neither was strictly a monofunctional compound. However, ethylene oxide has now been found to be moderately active, while Bird<sup>10</sup> has confirmed the earlier results of Rapoport. Consequently, there can be no doubt that at least some mono-epoxides are capable of acting as radiomimetic compounds within the limits of the present test.

**Ethylenimine series.** Ethylenimine itself has been found to be an efficient radiomimetic agent. In this

case, however, one cannot be sure that the compound is acting as a monofunctional reagent since, upon reaction, a primary amino-group is produced which might act as another functional centre in the molecule. This possibility is of special concern since putrescine (1:4 tetramethylene diamine) has been described as producing chromosome breakage. Although this substance has not been tested in the present work, cadaverine (1:5 pentamethylene diamine) has yielded negative results.

**Unsaturated compounds.** Two substances with strongly electrophilic double bonds have been examined, namely, acrolein (found to be inactive for *Drosophila* by Rapoport) and acrylonitrile. Both were found inactive. This is not perhaps surprising since both divinyl sulphone and proteanone, which showed radiomimetic behaviour at barely sub-lethal doses, and it has been found necessary in other cases to use considerably higher concentrations of compounds as compared with bi-functional compounds. The toxic concentrations of acrolein and acrylonitrile are such, however, that comparable concentrations cannot be employed.

**Miscellaneous.** Dimethyl sulphate has also been tested in view of Rapoport's findings and found to show a high degree of activity. Diazoethane, on the other hand, is inactive in this test; it can, however, only esterify unionized acids.

Except for the nitrogen mustards, where special conditions apply, the ratio of active concentrations as between mono- and poly-functional analogues shows the remarkably high value of about 1:5. Though these concentrations are admittedly not precise and do not necessarily bear a direct relation to that obtaining at the site of action, nevertheless the constancy of this difference is probably not without significance. It must be confessed that a satisfactory explanation is at present available to account for the disproportionate increase in activity that accompanies the insertion into the molecule of a second reactive group.

### Peroxides and Per-salts

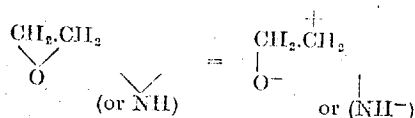
A purified specimen of tert.-butyl hydroperoxide (which is used as an initiator of polymerization reactions in the plastics industry) was found active in producing breakage and rearrangement of *Pha* chromosomes under the conditions employed, and incidentally the compound does not appear to be decomposed by catalase. Dieckoy, Cleland and Lon<sup>22</sup> have, in addition to this compound, employed aqueous mixtures of carbonyl compounds—formaldehyde and acetone—and hydrogen peroxide in their experiments with *Neurospora*. Formaldehyde may form dihydroxymethyl peroxide in aqueous hydrogen peroxide, and such a mixture was found in our experiments to produce a low incidence of chromosome breakage at just sub-lethal concentrations, controls performed with either component alone giving only negative results. It is conceivable that stabilization of naturally formed hydrogen peroxide may account for the results of Rapoport<sup>3</sup> and Auerbach<sup>23</sup> with formaldehyde.

The positive result obtained with tert.-butyl hydroperoxide (which has also been reported as producing erythema and vesication<sup>24</sup>) made it imperative to examine simple peroxy compounds in everyday use; sodium perborate, which is attacked by catalase, and sodium persulphate, which is not

were both found inactive. The inactivity of these and hydrogen peroxide itself suggests that the active principle may be an alkyl radical and not hydroxyl, especially since alkyl radicals may exist in equilibrium with the corresponding ion in solution.

It appears that compounds which will react readily under physiological conditions only with  $-SH$  and  $-NH_2$  groups, even though they be bifunctional, do not in general produce radiomimetic effects. Consequently, it is necessary to re-examine the properties of the two apparent exceptions—divinyl sulphone and protoanemonin—to ascertain whether they take part in other reactions. First, we may consider the basis of reactivity of the third class of compounds showing a high order of biological activity. It has been stated that these are all electrophilic reagents, this property being determined by their ability to yield a carbonium ion, that is, take up a form in which a positive charge resides on one of the carbon atoms. This is achieved in the mustards by ionization of the halogen atom, which occurs as a unimolecular reaction in aqueous solution and is facilitated by the electron-repelling character of the nitrogen or sulphur atom, as already described<sup>1</sup>. The carbonium ion can now react with water or with a nucleophilic centre; but the rate-determining step is the primary ionization, which is independent of the concentration of the second reagent. A full treatment of this subject can be found in the chemical literature, for example, Ogston<sup>24</sup> and Ross<sup>25</sup>.

The reaction of epoxides and ethylenimines is consistent with the assumption<sup>26</sup> that the ring opens with the formation of a carbonium ion, thus:



This likewise will react with water or a nucleophilic centre, and a hydrogen ion will add to  $O^+$  or  $NH^+$ . However, there is a contrast between these and the mustards in that the ring does not open except under the polarizing influence of a reactant. The reaction is thus bimolecular, and its rate determined by the concentration of reacting substrate; it might be expected that the two types would show differing degrees of selectivity in their action.

It can now be seen that the active unsaturated compounds have similar features. Thus, the electron-attracting group will cause a displacement of the double  $\pi$ -electrons of the double bond with the resulting polarization of the bond and the production of a positively-charged terminal carbon atom. The capacity of the double bonds to react with anions is confirmed by the fact that divinyl sulphone reacts with the thiosulphate anion, which has a very high rate of competition for the carbonium ion. As a further test of this principle, Ross (in the press) has examined the reactivity of some unsaturated aldehydes and esters toward thiosulphate and has shown that a significant reaction only occurs where the vinyl group is unsubstituted and immediately adjacent to the electron-attracting centre, for example, acrolein, acrylonitrile and acrylic ester, but not methacrylic ester, crotonaldehyde, crotonic ester or cinnamic aldehyde.

However, reaction with groups other than anions is strongly favoured since the double bond, being at all times polarized, is able to polarize and react with other groups in turn, especially  $-SH$  and  $-NH_2$ . Moreover, esters derived by reaction with acid anions are liable to be very unstable. Divinyl sulphone is thus one of the least active of the radiomimetic class and has a high toxicity. In the absence of experimental evidence, it is assumed that the activity of protoanemonin depends upon a similar mechanism. The very low activity of dichlorodiethyl sulphone must be related to the ease with which it loses hydrogen chloride to give divinyl sulphone (cf. Boursnoll *et al.*<sup>27</sup>).

So far, it appears that all the genetically active compounds discussed have the common property of reacting by a carbonium ion mechanism. If Ross's assumption, as already referred to, is correct, it seems difficult to avoid the conclusion that the biologically significant reaction is an esterification of ionized acids. The possibility of direct action with nucleoprotein aggregates in the resting nucleus has previously been suggested.

It remains to consider whether other radiomimetic or mutagenic agents can be accommodated within the same general plan. Work with peroxides is as yet in its early stages, and more will be said concerning this in the later paper. Nevertheless, it has already been proposed by Ross<sup>28</sup> that there are points of similarity between free radicals and carbonium ions.

Many of the compounds which Rapoport has claimed to be mutagenic, such as diazomethane, diethyl sulphate and, as we have seen, acrolein, react readily as esterifying agents. It is, however, evident that certain mutagens such as phenol<sup>29</sup>, urethane<sup>30,31</sup>, carcinogenic hydrocarbons,  $\beta$ -naphthylamine<sup>3</sup> and caffeine<sup>32</sup> fall into a different category both chemically and, in so far as their level of mutagenic activity is concerned, biologically. It is not out of the question, however, that some of them should be converted to reactive derivatives, for example, epoxides or peroxides, *in vivo*.

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### Summary

Tests with a considerable range of mono- and poly-functional alkylating and esterifying agents for radiomimetic activity in *Flies* have shown that only those compounds are active which are capable of reacting by a carbonium ion mechanism. While both mono- and poly-functional types are active, the latter show a very much higher degree of activity; no entirely satisfactory explanation of this fact has been proposed.

The results suggest that activity resides in the high degree of selectivity of reaction with anions which is manifested by the group, and that the significant reaction *in vivo* is probably an esterification. The radiomimetic activity of organic peroxides is also reported, and the suggestion offered that certain mutagens, for example, formaldehyde, may act through the intermediate formation of organic peroxides by reaction with naturally produced hydrogen peroxide. It is suspected that the activity of organic

peroxides lies in their ability to liberate alkyl rather than hydroxyl radicals.

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## The Oxidation of Acetylmethylcarbinol to Diacetyl in Butter Cultures

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## The Oxidation of Acetylmethylcarbinol to Diacetyl in Butter Cultures<sup>1</sup>

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Diacetyl, acetylmethylcarbinol, and 2,3-butyleneglycol constitute a series of compounds that is of special importance in butter cultures and, presumably, in butter manufactured with the use of culture. A satisfactory butter culture commonly contains relatively large quantities of acetylmethylcarbinol that are formed through the activity of the citric acid-fermenting streptococci normally present in the culture (2). Small portions of this acetylmethylcarbinol appear to be oxidized to diacetyl which is important from the standpoint of the aroma of the culture. Some of the acetylmethylcarbinol is also reduced to 2,3-butyleneglycol through the action of the citric acid-fermenting streptococci (1). The reduction is much slower in a butter culture than in a pure culture of one of the streptococci to which acetylmethylcarbinol has been added, and this relatively slow reduction is probably due to the inhibitory effect of the acid in the butter culture on these streptococci.

From the standpoint of the aroma of a butter culture, the oxidation of acetylmethylcarbinol to diacetyl is especially important since diacetyl, in the proper concentration, has an odor suggestive of fine butter, while highly purified acetylmethylcarbinol is odorless. Accordingly, an attempt was made to determine whether this oxidation is a direct chemical action or whether it is brought about through the activity of the citric acid-fermenting streptococci. The general procedure followed was to study the effect of carbon dioxide, hydrogen, nitrogen, and oxygen on the production of diacetyl (a) in acidified sterile milk to which acetylmethylcarbinol had been added, (b) in acidified cultures of the citric acid-fermenting streptococci, and (c) in butter cultures.

### METHODS

Diacetyl was determined as nickel dimethylglyoximate (3), using either a 200 or 400-gm. sample for analysis; carbon dioxide or nitrogen was bubbled through the system for some time before the distillation was begun, in order to remove oxygen which might produce diacetyl from acetylmethylcarbinol during the process. Acetylmethylcarbinol was also determined as nickel dimethylglyoximate by oxidizing the carbinol in a 200-gm. sample to diacetyl with ferric chloride and distilling it as diacetyl (3).

<sup>1</sup>Project 127 of the Iowa Agricultural Experiment Station.

In some cases small quantities of oxidizing reagents were added to the water solutions (without the use of a gas) in an attempt to oxidize the acetylmethylcarbinol. Hydrogen peroxide and potassium permanganate did not produce diacetyl in 48 hours at 21° C. Potassium dichromate yielded a trace of diacetyl but as much diacetyl was obtained when the mixture was distilled at once as when it was distilled after 48 hours which suggests that the diacetyl was produced during the distillation process rather than by the effect of the potassium dichromate at 21° C.

The effect of various gases on the production of diacetyl by the citric acid-fermenting organisms in milk was studied as follows: Lots of sterile skim milk were inoculated with the organism to be used and incubated at 21° C. After acidifying with citric (0.15 percent) and sulfuric (usually 0.30 percent) acids to a pH satisfactory for the rapid production of acetylmethylcarbinol, various gases were bubbled through the different lots. The usual determinations were made following 48 hours of incubation at 21° C. The data obtained are given in table 2.

In each trial there was some variation in the yields of acetylmethylcarbinol plus diacetyl with the various gases and in certain of the trials the variations were very large. None of the gases regularly gave either the highest or lowest production of the carbinol. There were relatively large variations in the yields of diacetyl and the largest yield was always obtained when oxygen had been bubbled through the culture while the second largest yield was always obtained with the culture exposed to the air. In a number of instances there was a comparatively low yield of acetylmethylcarbinol plus diacetyl with oxygen or exposure to air and at the same time the yield of diacetyl was relatively high. There was no regular variation in the yields of diacetyl with carbon dioxide, hydrogen, and nitrogen.

The data indicate that the production of diacetyl by the citric acid-fermenting streptococci was definitely modified by bubbling various gases through the cultures and that oxygen gave the largest yield of diacetyl, air gave the next largest yield, while carbon dioxide, hydrogen, and nitrogen gave relatively low yields.

Additional results on the effect of various gases on the production of diacetyl by the citric acid-fermenting organisms were obtained by inoculating lots of sterile skim milk with one of the organisms, incubating 24 hours at 21° C., acidifying some lots with sulfuric acid (0.37 or 0.38 percent) and other lots

TABLE 2. EFFECT OF VARIOUS GASES ON ACIDIFIED CULTURES OF THE CITRIC ACID-FERMENTING STREPTOCOCCI.

Sterile skim milk inoculated and held at 21° C. for 15 hours with citric acid (usually 0.30%) to give the desired pH then added; various gases bubbled through portions and containers sealed; analyses and ac. determined after 48 hours at 21° C.

Organism used	Hours incub. before adding acids	pH after adding acid	Gas bubbled through milk	gm. N. dimethylglyoxime (21° C.)	
				none added per 200 gm.	air per 200 gm.
9	24	3.1*	none	.0000	.0000
			CO <sub>2</sub>	.0000	.0000
			H <sub>2</sub>	.0000	.0000
			N <sub>2</sub>	.0000	.0000
			O <sub>2</sub>	.0420	.0076
34	24	3.9	none	.1153	.00175
			CO <sub>2</sub>	.1056	.0024
			H <sub>2</sub>	.1094	.0022
			N <sub>2</sub>	.0943	.00825
			O <sub>2</sub>		
49	24	3.9	none	.0901	.00375
			CO <sub>2</sub>	.0840	.00295
			H <sub>2</sub>	.0912	.00315
			N <sub>2</sub>	.0953	.0033
			O <sub>2</sub>	.0738	.00505
146	24	3.8	none	.0822	.0062
			CO <sub>2</sub>	.0876	.0035
			H <sub>2</sub>	.0850	.00375
			N <sub>2</sub>	.0869	.01075
			O <sub>2</sub>		
146	24	3.9	none	.0771	.0080
			CO <sub>2</sub>	.0935	.0077
			H <sub>2</sub>	.0975	.00785
			N <sub>2</sub>	.1010	.00735
			O <sub>2</sub>	.0881	.0125
29	40		none	.0653	.0094
			CO <sub>2</sub>	.1194	.0513
			O <sub>2</sub>	.0771	.0139
37	40		none	.0431	.0041
			CO <sub>2</sub>	.0673	.0013
			O <sub>2</sub>	.0081	.0122
37	40		none	.0916	.0043
			CO <sub>2</sub>	.0684	.0019
			O <sub>2</sub>	.0790	.0082

\*0.40% sulfuric used to get low pH.

with citric (0.15 percent) and sulfuric (0.31 or 0.32 percent) acids, bubbling various gases through the different lots and then making the usual analyses after 48 hours at 21° C. Table 3 gives the data obtained with two organisms.

With each organism the addition of citric acid gave the expected increase in the production of acetylmethylcarbinol plus diacetyl. Either with or without the addition of citric acid there was some variation in the yields of acetylmethylcarbinol plus diacetyl in the presence of the various gases, but these were not large except in the case of one of the organisms without added citric acid. There were relatively large variations

The pH determinations were made electrometrically, using quinhydrone.

When gas was to be bubbled through milk, water, or a culture, the material was placed in a bottle fitted with a stopper carrying two glass tubes. One of the tubes, which was used as the inlet tube, led nearly to the bottom of the bottle while the other, or outlet tube, terminated a very short distance below the stopper. Gas from a cylinder was bubbled through the material rather rapidly for about 10 minutes; although the material was agitated by the current of gas, it was occasionally shaken. When the treatment was complete, clamps were placed on the rubber connections leading to the glass tubes. A few lots of milk foamed considerably but as a rule no difficulty was encountered.

Commercial acetylmethylcarbinol was purified by washing with cold anhydrous ether, as suggested by Stahly and Werkman (5).

#### EXPERIMENTAL.

The attempts to oxidize acetylmethylcarbinol to diacetyl in milk, without the action of bacteria, were carried out as follows: A quantity of sterile skim milk was acidified to a pH satisfactory for the rapid production of acetylmethylcarbinol when the citric acid-fermenting streptococci are present. Usually a mixture of citric (0.15 percent) and sulfuric (0.28 to 0.30 percent) acids was employed to give a pH of 3.8 or 3.9 because under these conditions the citric acid-fermenting organisms produce the carbinol in relatively large amounts; in one case lactic acid was used to give about the pH found in a ripened butter culture. A solution of acetylmethylcarbinol was then added and various gases bubbled through different portions of the milk. After 48 to 72 hours at 21° C. determinations of acetylmethylcarbinol plus diacetyl and of diacetyl were made. Table 1 presents representative data.

In each trial the quantities of acetylmethylcarbinol plus diacetyl found in the various portions of milk were essentially the same. When commercial or partially purified acetylmethylcarbinol was added to the milk, diacetyl was found on distillation, but the quantities in the different portions of milk used in a trial showed no regular variations and bubbling carbon dioxide, hydrogen, nitrogen or oxygen through the milk did not significantly influence the amount. With the addition of purified acetylmethylcarbinol to the milk, appreciable quantities of diacetyl were not obtained with any of the gases.

The results show that when acetylmethylcarbinol was added to milk there was no appreciable formation of diacetyl in 48

TABLE 1. EFFECT OF VARIOUS GASES ON ACETYL METHYLCARBINOL IN ACIDIFIED, STERILE SKIMMILK.

Acetylmethylcarbinol and acid added to sterile skim milk; various gases bubbled through different portions and containers sealed; amc = amc and acs determined after bubbling 48 or 72 hours at 21° C.

amc used	Acid used	pH at once	Hours held	Gas bubbled through milk	gm. N-acetylhydrazine hydrate 1 to 2	
					amc + acs per 200 gm.	acs per 200 gm.
Commercial	0.15% citric .29% sulfuric	3.8	48	none	.1241	.0680
				CO <sub>2</sub>	.1271	.0881
				H <sub>2</sub>	.1274	.0815
				O <sub>2</sub>	.1247	.0670
Commercial	0.15% citric .30% sulfuric	3.8	48	none	.1222	.0650
				CO <sub>2</sub>	.1195	.0628
				H <sub>2</sub>	.1167	.0685
				O <sub>2</sub>	.1176	.0675
Partially purified	0.15% citric .30% sulfuric	3.9	48	none	.1120	.0655
				CO <sub>2</sub>	.1163	.0685
				H <sub>2</sub>	.1177	.0633
				N <sub>2</sub>	.1079	.0625
Purified	0.15% citric .30% sulfuric	3.9	48	none	.1110	.0632
				CO <sub>2</sub>	.1386	none
				H <sub>2</sub>	.1437	none
				N <sub>2</sub>	.1406	none
Purified	0.15% citric .28% sulfuric	3.9	48	O <sub>2</sub>	.1343	none
				none	.1364	none
				CO <sub>2</sub>	.0485	none
				H <sub>2</sub>	.0492	none
Purified	0.15% citric .30% sulfuric	3.8	72	N <sub>2</sub>	.0469	none
				O <sub>2</sub>	.0491	none
				none	.0445	none
				CO <sub>2</sub>	.1989	none
Purified	0.70% lactic	4.4	72	H <sub>2</sub>	.1953	none
				CO <sub>2</sub>	.1872	none
				N <sub>2</sub>	.1896	none
				O <sub>2</sub>	.1822	none
Purified	0.70% lactic	4.4	72	none	.1486	none
				CO <sub>2</sub>	.1470	none
				H <sub>2</sub>	.1475	none
				N <sub>2</sub>	.1477	none
Purified	0.70% lactic	4.4	72	O <sub>2</sub>	.1458	none
				none		none
				CO <sub>2</sub>		none
				H <sub>2</sub>		none

\*amc = acetylmethylcarbinol.  
acs = diacetyl.

to 72 hours at an acidity and a temperature satisfactory for the rapid production of the carbinol when the citric acid-fermenting streptococci are present.

Attempts to oxidize acetylmethylcarbinol to diacetyl in a short time were also carried out in water. Acetylmethylcarbinol was added to sterile water, various gases were bubbled through different portions and the acetylmethylcarbinol plus diacetyl and the diacetyl determined after 48 hours at 21° C. When purified acetylmethylcarbinol was used, diacetyl was not found with carbon dioxide, hydrogen, nitrogen, oxygen, or in the controls.

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TABLE 3. EFFECT OF VARIOUS GASES ON CULTURES OF THE CITRIC ACID-FERMENTING STREPTOCOCCI ACIDIFIED WITH SULFURIC ACID OR WITH CITRIC AND SULFURIC ACIDS.

Sterile skim-milk inoculated and held 24 hours at 21°C.; sulfuric acid added to some portions and citric and sulfuric acids to others; various gases bubbled through different portions and containers sealed; ams, ams and ams determined after holding 48 hours at 21°C.

Organism used	Acidity adjusted with	pH after adding acid	Gas bubbled through milk	gm. Ni dimethylglyoximate 24 to	
				ams—ams per 200 gm.	ams per 200 gm.
D2	0.38% sulfuric	3.9	none	0.0364	trace
			CO <sub>2</sub>	0.0364	trace
			H <sub>2</sub>	0.0354	trace
			O <sub>2</sub>	0.0302	0.0345
	0.15% citric 0.32% sulfuric	3.9	none	0.0112	0.0067
			CO <sub>2</sub>	0.026	trace
			H <sub>2</sub>	0.081	trace
			O <sub>2</sub>	0.071	0.015
20	0.37% sulfuric	3.9	none	0.048	0.0018
			CO <sub>2</sub>	0.090	0.007
			H <sub>2</sub>	0.075	trace
			O <sub>2</sub>	0.059	0.003
	0.15% citric 0.31% sulfuric	3.9	none	0.079	0.0001
			CO <sub>2</sub>	0.076	0.002
			H <sub>2</sub>	0.074	0.004
			O <sub>2</sub>	0.082	0.0181

in the yields of diacetyl. The largest yield was regularly obtained when oxygen had been bubbled through the culture and in three of the four cases exposure to air gave a strikingly larger yield than carbon dioxide or hydrogen. Commonly there was more diacetyl present in the cultures containing a relatively large amount of acetylmethylcarbinol plus diacetyl, as the result of the addition of citric acid, than in the cultures containing a comparatively small amount of the carbinol. The differences were particularly striking with oxygen and exposure to air.

The effect of bubbling various gases through cultures of one of the citric acid-fermenting organisms on the production of diacetyl when citric acid alone was used to acidify the cultures is shown in table 4.

There was some variation in the yields of acetylmethylcarbinol plus diacetyl with the different gases and a very definite variation in the yields of diacetyl. The largest yield of diacetyl was obtained with oxygen and the next largest yield with exposure to air, while carbon dioxide, hydrogen and nitrogen did not give appreciable quantities of diacetyl. The relatively high yield of diacetyl when oxygen was bubbled through the culture accompanied a comparatively low yield of acetylmethylcarbinol plus diacetyl.

When acetylmethylcarbinol is added to an unacidified tomato

TABLE 4. EFFECT OF VARIOUS GASES ON A CULTURE OF ONE OF THE CITRIC ACID-FERMENTING STREPTOCOCCI ACIDIFIED WITH CITRIC ACID.

Sterile skim-milk inoculated and held 18 hours at 21°C.; 0.7% citric acid added; various gases bubbled through different portions and containers sealed; ams, ams and ams determined after holding 48 hours at 21°C.

Organism used	pH after adding acid	Gas bubbled through milk	gm. Ni dimethylglyoximate 24 to	
			ams—ams per 200 gm.	ams per 200 gm.
32	3.7	none	0.0729	0.0026
		CO <sub>2</sub>	0.0818	none
		H <sub>2</sub>	0.0827	none
		N <sub>2</sub>	0.0772	none
		O <sub>2</sub>	0.0665	0.009
			0.041	0.007

broth or milk culture of one of the citric acid-fermenting organisms, there is a rapid reduction to 2,3-butyleneglycol and, if diacetyl is added, there is a reduction to the glycol or to the carbinol (1). This general relationship makes it improbable that added acetylmethylcarbinol would be oxidized to diacetyl by the organisms in an unacidified culture but a few trials were carried out in an attempt to find small quantities of diacetyl in a culture in which the main change was a reduction of the carbinol. Both tomato broth and milk were used with carbon dioxide, hydrogen, nitrogen, oxygen and without added gas but appreciable quantities of diacetyl were never detected. The acetylmethylcarbinol largely disappeared during the holding period. Trials were also carried out with tomato broth, using cultures that had been acidified with sulfuric acid to a pH of about 3.9 in order to delay the reduction by the organisms, but appreciable quantities of diacetyl were not found; comparable trials with milk were not used because the presence of citric acid in the milk would have resulted in a production of acetylmethylcarbinol plus diacetyl at a low pH.

The results obtained with pure cultures of the citric acid-fermenting organisms are not directly applicable to butter cultures because (a) the typical lactic acid-producing organisms in a butter culture may influence the changes that occur and (b) there are smaller numbers of the citric acid-fermenting organisms per milliliter in a butter culture than in the pure cultures used. The action of various gases on butter cultures was studied as follows: Pasteurized skim-milk was inoculated with a butter culture and 0.15 percent citric acid added. Various gases were then bubbled through different portions, the cultures held at 21°C. for 15 to 20 hours and the determinations made after holding the ripened cultures in a refrigerator for 19 to 24 hours. Table 5 gives the results obtained.

TABLE 5. EFFECT OF VARIOUS GASES ON BUTTER CULTURES.

Pasteurized skimmilk inoculated and 0.1% citric acid added; various gases bubbled through different portions and containers sealed; held from 15 to 20 hours at 21° C.; removed to refrigerator and analysed and ac. determined after 19 to 24 hours.

Butter culture used	Gas bubbled through milk	pH on analysis	gm. N: diacetyl plus acetate % to	
			none added per 200 gm.	ac. per 400 gm.
115	none	4.4	0.0747	none
	CO <sub>2</sub>	4.1	0.0172	none
	H <sub>2</sub>	4.5	0.0757	none
	N <sub>2</sub>	4.4	0.0290	none
	O <sub>2</sub>	4.5	0.0561	none
	O <sub>2</sub>	4.5	0.0551	0.0005
122-F	none	4.4	0.0878	none
	CO <sub>2</sub>	4.5	0.0886	none
	H <sub>2</sub>	4.5	0.0876	none
	N <sub>2</sub>	4.4	0.0892	none
	O <sub>2</sub>	4.5	0.0882	0.0025
	O <sub>2</sub>	4.5	0.0882	0.0025
122-F	none	4.4	0.0843	0.0005
	CO <sub>2</sub>	4.1	0.0794	0.015
	H <sub>2</sub>	4.5	0.0884	0.0035
	N <sub>2</sub>	4.5	0.0900	0.005
	O <sub>2</sub>	4.5	0.0828	0.022
	O <sub>2</sub>	4.4	0.0885	0.015
116	none	4.4	0.0911	0.017
	CO <sub>2</sub>	4.4	0.0885	0.015
	H <sub>2</sub>	4.5	0.0909	0.017
	N <sub>2</sub>	4.5	0.0928	0.018
	O <sub>2</sub>	4.5	0.094	0.021
	O <sub>2</sub>	4.4	0.0909	0.0205
122-15	none	4.4	0.0565	0.020
	O <sub>2</sub>	4.4	0.056	0.025
	O <sub>2</sub>	4.4	0.055	0.025
232	none	4.4	0.111	0.029
	CO <sub>2</sub>	4.4	0.0871	0.026
	O <sub>2</sub>	4.5	0.073	0.034
M1	none	4.3	0.050	none
	O <sub>2</sub>	4.4	0.042	0.0085

The variations in the yields of acetylmethylcarbinol plus diacetyl with the various gases were rather large, particularly in certain of the trials. There were also variations in the yields of diacetyl, with the portions through which oxygen had been bubbled regularly showing the highest yields. The portions with which carbon dioxide, hydrogen, nitrogen or air had been used frequently did not show appreciable quantities of diacetyl but, when they did, air gave higher yields than carbon dioxide, hydrogen or nitrogen. The variations in the yields of diacetyl in the different trials are rather striking. They cannot be attributed to differences in the butter cultures employed since one of the trials with 122-F gave appreciable quantities of diacetyl only when oxygen was used while the other gave appreciable quantities under all the conditions and, with oxygen, yielded much more diacetyl than in the other trial.

The action of oxygen on butter cultures made with various amounts of citric acid was studied by inoculating pasteurized skimmilk, dividing it, adding different amounts of citric acid to different portions and bubbling oxygen through some portions; after incubation the cultures were removed to the refrigerator and the usual determinations made after 24 hours. The data obtained are given in table 6.

TABLE 6. EFFECT OF OXYGEN ON BUTTER CULTURE MADE WITH VARIOUS AMOUNTS OF CITRIC ACID ADDED TO THE MILK.

Pasteurized skimmilk inoculated with culture 122; divided and various amounts of citric acid added to different portions; oxygen bubbled through certain portions and containers sealed; held 20 hours at 21° C.; removed to refrigerator and analysed and ac. determined after 24 hours.

Citric acid added	N: gas bubbled through		Oxygen bubbled through	
	gm. N: diacetyl plus acetate % to		gm. N: diacetyl plus acetate % to	
	none added per 200 gm.	ac. per 400 gm.	none added per 200 gm.	ac. per 400 gm.
none	0.0414	none	0.0254	none
0.1%	0.049	none	0.029	0.017
.2	0.048	none	0.031	0.020
.3	0.045	none	0.039	0.025

Either with or without oxygen, the addition of the increasing amounts of citric acid gave a progressive increase in the yields of acetylmethylcarbinol plus diacetyl. Without oxygen no appreciable production of diacetyl occurred while with oxygen appreciable quantities were obtained in all cases, except the control to which no citric acid had been added, and the yields of diacetyl increased as the yields of acetylmethylcarbinol increased.

The influence of the addition of citric acid to the milk intended for butter culture when oxygen was bubbled through the culture was studied by inoculating pasteurized skimmilk and adding citric acid to some portions; after incubating 5 hours at 21° C. citric acid was added to the remaining portions and oxygen bubbled through all the lots; following additional incubation the cultures were removed to the refrigerator and subjected to the usual analyses after 6 hours and again after 24 hours. The butter culture used was one which appeared to be especially active in the production of diacetyl. Table 7 presents the data obtained.

The addition of citric acid regularly gave an increase in the yields of acetylmethylcarbinol plus diacetyl that was usually roughly proportional to the amounts of citric acid used. With either 0.2 or 0.3 percent citric acid the addition of the acid at the time of inoculation gave a larger yield of acetylmethyl-



TABLE 7. EFFECT OF VARIOUS AMOUNTS OF CITRIC ACID ON A BUTTER CULTURE WHEN OXYGEN WAS BUBBLED THROUGH.

Pasteurized skim milk inoculated with butter culture 232; various amounts of citric acid added to some portions at time of inoculation and to others 5 hours later; oxygen bubbled through all portions 5 hours after inoculation; incubated 21 hours at 21°C.; removed to refrigerator and analyzed and analyzed again after 6 hours and again after 24 hours.

Citric acid added		1st analysis		2nd analysis	
		mg. Ni dimethylglyoximate > to gm. Ni dimethylglyoximate > to			
At time of inoc.	5 hours after inoc.	analyses per 200 gm.	avg. per 400 gm.	analyses per 200 gm.	avg. per 400 gm.
none	none	0.0112	0.0035	0.0124	0.0035
0.1%		.0046	.0035	.0070	.0035
.2		.0054	.0038	.1114	.0038
.3		.1830	.0068	.1518	.0055
	0.1%	.0046	.0034	.0834	.0035
	.2	.0708	.0035	.1050	.0035
	.3	.0746	.0071	.1062	.0034

carbinol plus diacetyl than the addition 5 hours after inoculation. Accompanying the increased production of acetylmethylcarbinol plus diacetyl there was an increase in the production of diacetyl that was especially striking in the comparison of the effects of 0.2 and 0.3 percent citric acid. The results also show the influence of holding a ripened butter culture in the refrigerator for some time. In all cases, more acetylmethylcarbinol plus diacetyl was found at the second analysis than at the first and as a rule the difference was striking; the yield of the control culture at the second analysis was unusually high for a culture made without added citric acid. The increased production of acetylmethylcarbinol plus diacetyl on extended holding in the refrigerator was regularly accompanied by an increase in the diacetyl content.

Because of the possibility that *Streptococcus lactis* is involved in the oxidation of acetylmethylcarbinol in a butter culture, a number of trials were carried out with this species. The procedure used was to add the carbinol to sterilized milk, inoculate with *S. lactis* and then divide the milk into portions and pass carbon dioxide through one portion and oxygen through another; a control, which was not treated with gas, was also employed. In addition, each *S. lactis* culture was grown in milk to which no carbinol had been added. Diacetyl and acetylmethylcarbinol plus diacetyl were determined at the time of inoculation and again after 24 hours at 21°C. (when coagulation was complete) plus 24 hours in the refrigerator. There was no evidence of an oxidation of the carbinol to diacetyl in any of the trials. With one of the *S. lactis* cultures, a trace of diacetyl was produced in milk alone.

## DISCUSSION OF RESULTS

The results presented indicate that the formation of diacetyl from acetylmethylcarbinol in a pure culture of a citric acid-fermenting *Streptococcus*, or in a butter culture, is due to the activity of organisms rather than to a simple chemical oxidation. In the case of a butter culture, *S. lactis* apparently is of no importance in this connection. Although accurate comparisons cannot be made, there appears to have been a relatively larger production of diacetyl in pure cultures of the citric acid-fermenting streptococci than in butter cultures. This may have been due to the comparatively small number of citric acid fermenters in the butter cultures, but the presence of *S. lactis* is a possible factor also. It should be noted that, because of the difficulties involved in the determination of extremely small amounts of diacetyl, the butter cultures in which appreciable quantities of diacetyl were not found may have contained enough of this compound to definitely influence the odor.

While the results obtained cannot be applied directly to butter, they suggest that in this product acetylmethylcarbinol is oxidized to diacetyl through the activity of organisms. This general relationship is in agreement with the many observations on the comparative flavor development in unsalted and normally salted butter. With unsalted butter, in which the butter culture organisms can multiply actively (4), a conspicuous increase in the volume of the desirable flavor is frequently noted when the butter is held at a temperature that is at all favorable for the growth of butter culture organisms, while with normally salted butter, in which the culture organisms fail to multiply, there is usually no significant increase in the desirable flavor. In butter with a relatively low salt content the culture organisms would be expected to multiply to some extent and such butter may show a definite increase in the volume of the desirable flavor.

If the action of butter culture organisms is necessary for the oxidation of acetylmethylcarbinol to diacetyl in butter, the presence of the carbinol in unsalted butter would be of questionable value, from the standpoint of flavor development, unless butter culture were used in the manufacture, and the same is true of its presence in butter containing culture together with sufficient salt to prevent the development of the culture organisms. Acetylmethylcarbinol may be present in butter made without culture as a result of bacterial action in the cream or the actual addition to either cream or butter. It appears that when conditions are unsatisfactory for the growth of the butter culture organisms in butter, the oxidation of acetylmethyl-

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carbinol to diacetyl must occur in the butter culture or in the cream.

The conspicuous odor that is often noted in commercial preparations of acetylmethylcarbinol is commonly regarded as due to diacetyl formed through an oxidation of the carbinol. Diacetyl can be detected readily in such preparations by the usual analytical procedures. The formation of diacetyl in preparations of acetylmethylcarbinol without the action of organisms is not necessarily at variance with the idea that organisms are required for such an oxidation in a butter culture. The oxygen of the air may be more effective in oxidizing acetylmethylcarbinol in a concentrated form, especially in the absence of the complex compounds found in milk. However, it is difficult to understand how extensive oxidation could occur with the limited oxygen supply available in some of the containers used for preparations of acetylmethylcarbinol. A possible arrangement of two molecules of the carbinol so as to yield one molecule of diacetyl and one of 2,3-butylene glycol is another possible source of diacetyl. It should be noted also that some preparations of purified acetylmethylcarbinol remain free from the odor of diacetyl for extended periods of time even with an abundant air supply.

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## MARSCHALL DIVISION

MILES MILES LABORATORIES, INC.

October 16, 1973

P.O. BOX 592, MADISON, WISCONSIN 53701

(608) 257-9246

Dr. Humphrey Sassoon  
Tracor Jitco, Inc.  
1300 East Guide Drive  
Rockville, Maryland 20851

Dear Dr. Sassoon:

Your letter of September 19, 1973 regarding starter distillates has been sent to my attention as the preparation of these products take place at the Madison Operations.

You are correct in the evaluation of the material as a steam distillate from the various organisms you have identified.

Also a product simply termed "starter flavor" is developed which may be made entirely from a blend of chemical compounds or in conjunction with a pure starter distillate and then standardized to exact diacetyl levels. Generally the amount of material used in flavoring food products is such as to give a level of not more than 2 to 4 PPM diacetyl in the finished product.

We are not a basic manufacturer of diacetyl and therefore cannot supply you with data about this product.

We will be pleased to supply you with any additional information needed.

Very truly yours,

MARSCHALL DIVISION  
MILES LABORATORIES, INC.

*V.W. Christensen*  
V.W. Christensen  
Manager, Madison Operations

VWC/lat

Inactivation of Influenza Viruses by  
 $\alpha$ -Dicarbonyl Compounds\*

H. Mücke and M. Sprössig  
(From the Institute for Medical Microbiology  
of the Erfurt Medical Academy-  
Director: Prof. Dr. M. Sprössig)

The chemotherapy of virus infections is still in its very early stages of application. A certain measure of success in man has been achieved so far only in the prevention of smallpox in contacts with N-methylisatin- $\beta$ -thio-semicarbazone, in the treatment of certain eye diseases caused by viruses with iododesoxyuridine, and in a still disputed manner during the treatment of flu (influenza) with ABOB biguanide and amantadin.

In order to make a contribution to the very difficult problem of a chemotherapy of virus infections, it is therefore necessary to carry out further experiments on a broad basis, and finally also in order to obtain a progressive insight into the principles and mode of action of such treatment.

In this article, tests on influenza viruses with a certain group of compounds will be reported, which, at least from a theoretical standpoint, are of great interest.

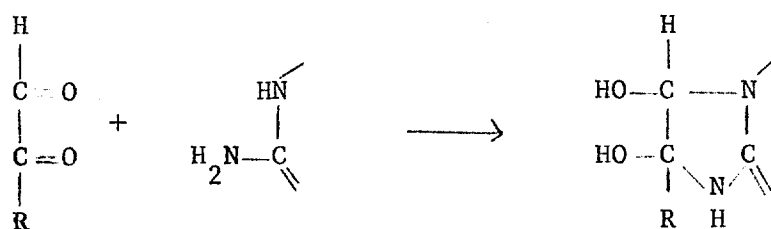
American authors reported in 1957 that glyoxals, i.e. definite and very reactive chemical compounds, were able to inhibit the multiplication of influenza and Newcastle disease viruses in embryo-containing eggs. Later, these compounds were also found to possess virusidal, i.e. virus-inactivating, properties. This action is only very seldom found in other classes of virus-active substances, and for this reason a direct reaction of glyoxals with a virus was assumed.

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\* Report presented at the Symposium on Disinfectants and Antiseptic Products on 24-25 September 1965 in Greifswald.

In order to confirm this assumption of a direct action on the virus, Staehelin (1959) investigated in a consequential (consistent) way the reaction of glyoxals with nucleotides, which constitute the monomeric cleavage products of nucleic acids, since it is known that the infectiosity of a virus is connected with the virus nucleic acid. Spectrophotometric studies of this reaction by Staehelin made it very probable that glyoxals react specifically with the guanine bases of ribonucleic acid under formation of stable five-ring structures, as shown schematically in formula group I.

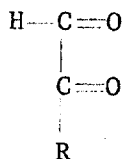
Studies carried out later with a radioactively-labeled glyoxal also supported the inactivation reaction with guanine described here.



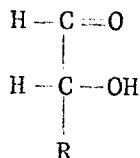
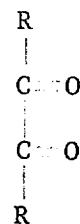
Formula group I

It should be noted in this connection that guanine is one of the four possible bases which, along with adenine, cytosine and uracil, is bound to ribose in ribonucleic acid.

At the beginning of our studies on the inactivation of influenza viruses, we tested some typical glyoxal compounds and compared their action with the structurally related glycerol aldehyde, an  $\alpha$ -hydroxy aldehyde, and the also structurally very closely related simplest  $\alpha$ -diketone, diacetyl (see Formula group II).



glyoxal derivative

 $\alpha$ -hydroxyaldehyde $\alpha$ -Diketone

## Formula group II

For the purpose of virus inactivation, 0.9 ml of a solution of the substance, containing 1 mg (if possible in Hanks' solution), was added to 0.1 ml of virus-containing allantoic fluid. After different times of reaction of the test substances with the virus at room temperature, the material was diluted to a  $10^{-3}$  -  $10^{-9}$  concentration. Then, 11-day old incubated eggs were inoculated with 0.25 ml of these diluted solutions, whereby 6 eggs were used for each dilution stage. As control tests with exembryonated (embryo-free ?) eggs showed, a concentration range was used in this case, which excluded any possible aftereffect on the virus or damage to the host cell. The determination of ID<sub>50</sub> was done according to Reed and Muench.

Results of these first comparison studies are shown in Fig. 1, whereby we shall not consider at first the two upper curves shown in this figure. The time periods during which the substances act on the virus, at room temperature and a concentration of 1000 microgram/ml, are plotted along the abscissa.

The remaining active virus residue is plotted along the ordinate axis of Fig. 1, and we can see from the course of the inactivation curves obtained that the time drop of the ID<sub>50</sub> is quite different for the individual substances tested. Phenylglyoxal hydrate was the most active product here, since already after 15 minutes the remaining active virus residue had shrunk to 1 millionth of the original amount. A progressively lower (weaker) activity

was exhibited by 4-biphenyl glyoxal bisulfite and glyoxal bisulfite, which is understandable in the light of the inactivation reaction described above. In the bisulfite compounds, the reactive aldehyde group is namely occupied by  $\text{NaHSO}_3$  and thus protected from oxidation and polymerization; this fact, however, should have a negative effect on the velocity (rate) of the addition reaction to guanine. In addition, we must keep in mind the fact that in both these products the  $\text{NaHSO}_3$ , which is inactive by itself, is present in bound form. This reduces the concentration of the actually active substance, since 1000 micrograms/ml always acted upon the virus.

Of particular interest to us was the relatively good action (effect), with a good cell tolerance, of the 1,2-diketone, diacetyl, indicated in Fig. 1 by a dot-dash line, since  $\alpha$ -diketones were used by us for the first time in such studies. In contrast, the 1,3-diketone, acetylacetone, was almost inactive. This shows that a neighboring position of the two carbonyl groups is necessary for virus inactivation, since the two carbonyl groups in acetylacetone are separated by a methylene bridge. Glycerol aldehyde, as an  $\alpha$ -hydroxyaldehyde, is essentially similar in its action to diacetyl. The poor action (effect) exhibited by polymeric glyoxal can be explained by the saturation of the active aldehyde groups in the polymer molecule.

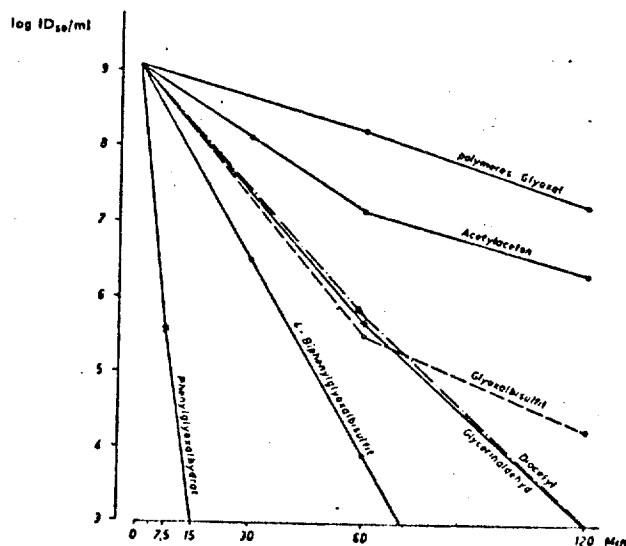


Figure 1

Before we discuss the in vitro testing of further  $\alpha$ -diketones, allow me to make a few incidental remarks. As we began to expand our studies to cover a larger number of  $\alpha$ -diketones, we were hoping from the very start to find products here which would be effective not only in vitro, but with some therapeutic success could perhaps be achieved in animal tests. The glyoxals which were highly active in vitro unfortunately were found to be noneffective in vivo, with the exception of some biphenylglyoxals, a group of products on which extensive work had been carried out by Italian researchers associated with Magrassi and Cavallini. The inactivity of glyoxals in animal tests is perhaps due to a large extent to the high chemical reactivity and instability of these compounds, i.e. their easy oxidizability and addition capability. In the tests performed by us, the considerably more stable  $\alpha$ -diketones exhibited, according to our concepts, although not a general, but still a slight and reproducible measure of therapeutic success with individual compounds on mice infected with influenza virus. Among other things, animal tests have the great advantage, as compared to in vitro testing, in that insoluble compounds can also be tested.

On the basis of the results showing that diacetyl is active against viruses, further available  $\alpha$ -diketones which are soluble or can be made soluble were investigated by us in in-vitro tests. Some results of these tests are shown in Fig. 2. For comparison, phenylglyoxal hydrate, the compound which had the greatest activity in Fig. 1, is shown again in Fig. 2 by means of a broken line. It was also found here that the degree of activity depends on the structure. Cyclohexane hexone and 1,2-naphthoquinone (also known as  $\beta$ -naphthoquinone) were found to be very active, and their activity was



even greater than that of phenylglyoxal hydrate. Whereas cyclohexane hex-one is relatively easily soluble in water, has practically no effect on cellular respiration in the Warburg test and is well tolerated in animal tests even at high dosages, 1,2-naphthoquinone was found to have a considerable cytotoxicity in the Warburg and animal tests, and, in addition, had to be dissolved in 30% methanol to act upon the virus, in view of its low water solubility. Control studies showed that 30% methanol alone is practically inactive under the same test conditions. Conversion of 1,2-naphthoquinone into a water-soluble form by introduction of a sulfonic acid group resulted in an almost complete loss of activity. Animal testing of this compound, which has become well-tolerated as a result of sulfonation, has not yet been carried out.

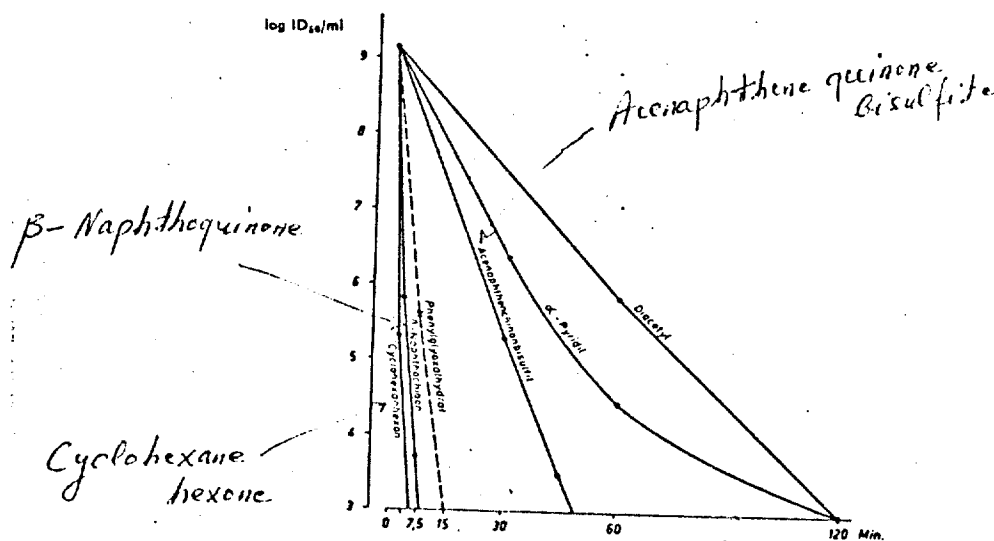


Figure 2

The two  $\alpha$ -diketones, acenaphthene quinone and phenanthrene quinone, have a very low water solubility and cannot be tested in this form. In both

cases, however, it is possible to prepare the readily water-soluble bisulfite compounds. As can be seen from the curve trajectory of acenaphthene quinone bisulfite, this compound exhibits a considerable activity. The phenanthrene quinone bisulfite compound apparently has a similar behavior; however, an exact (accurate) testing of this compound is not possible because it breaks down very rapidly into its components in aqueous solution, whereby a precipitate of insoluble phenanthrene quinone is obtained.

In animal testing, the low solubility is no obstacle, and we were able to achieve the best therapeutic success so far with acenaphthene quinone in this group of products considered at the present time.

The heterocyclic  $\alpha$ -diketone,  $\alpha$ -pyridil, exhibits a still considerable activity in vitro, although methanol had to be used as a solvent here. The heterocyclic compound, furil, has a very low solubility and therefore cannot be accurately tested in this manner. So far, we have been unable to obtain any noticeable activity in vitro with purely aromatic  $\alpha$ -diketones.

Further, cyclohexane hexone and diacetyl proved to be active in tests on virus-infected mice. The corresponding curves of the results obtained in vitro are widely separated in Fig. 2, and one could expect, after an unbiased examination of these curves, that cyclohexane hexone would also be superior in animal tests. However, this is not the case. Both compounds practically always exhibited the same therapeutic effect. Since animal tests have not yet been completed, we shall not describe here the details of such tests.

The high in vitro activity of cyclohexane hexone, which is accompanied by a remarkably good cell tolerance, can be derived from the structural configuration of this compound. Here, the entire hydroaromatic ring is filled with keto groups, and thus a concentration of active principles is present. For this reason, we have included this rather curious compound in our studies,

and we found that our expectations, derived from the formula, were confirmed.

The studies reported here have shown that, in addition to glyoxals,  $\alpha$ -diketones, which have not yet been investigated in this connection, can also inactivate influenza viruses. In addition,  $\alpha$ -diketones, as a rule, are much more stable and better tolerated by cells, with some members of this group, certain therapeutic effects can be achieved on virus-infected mice. In addition, some of these compounds can also be of interest in the practical application as virus-inactivating agents, for example, in the preparation of vaccines.

#### Discussion

Mr. Profft: I find the results reported here very interesting. What is the behavior of glycerol aldehyde, which is known to be active against mouse ascites? I would also like to suggest that flucrenone be included in such studies, since fluorenone derivatives are known to possess, in part, a good pharmacological action.

Answer: Glycerol aldehyde, which has assumed interest recently as a result of tests on the so-called multiple-step chemotherapy of cancer, shows, as was already reported, about the same activity as diacetyl in in-vitro tests, and thus has a quite good virus-inactivating action. Unfortunately, in mouse tests carried out by us so far, no signs showing that this compound can affect the course of an influenza virus infection have been found.

Mr. Mach: In your opinion, do the tested compounds act only on the guanine of the virus nucleic acids or, during virus replication, on the nucleic acid metabolism of the host cell?

Answer: The action of these compounds appears to be primarily directed at the virus nucleic acid, since we found that some highly active compounds have practically no effect, or only a slight effect, on the cellular respiration of the host cells.

Mr. Häussler: Sugar osones, as easily available aliphatic diketones, should be included in the testing.

Mr. Menzel: Starting from the fact that the virus inhibiting (virucidal) effect in vivo is clearly affected by the time relations between infection and the start of therapy, I would like to ask when was therapy started?

Mr. Mücke: The best course is a prophylactic or simultaneous (concurrent) administration. It is still possible to start therapy 3-4 hours after the infection.

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Aus dem Institut für Medizinische Mikrobiologie der Medizinischen Akademie Erfurt  
(Direktor: Prof. Dr. M. Sprößig)

# Über die Inaktivierung von Influenzaviren durch $\alpha$ -Dicarbonylverbindungen<sup>1)</sup>

von

H. Mücke und M. Sprößig

Mit 2 Abbildungen

(Eingegangen am 3. August 1966)

Die Chemotherapie der Virusinfektionen steckt noch in den allerersten Anfängen. Gewisse Erfolge beim Menschen sind bisher nur bei der Pockenprophylaxe von Kontaktpersonen mit dem N-Methylisatin- $\beta$ -thiosemicarbazon, bei der Behandlung bestimmter virusbedingter Augenerkrankungen mit Joddesoxyuridin und noch etwas umstritten bei der Grippebehandlung mit dem Biguanid ABOB und mit Amantadin erzielt worden.

Um zur Lösung des sehr schwierigen Problems einer Chemotherapie der Virusinfektionen beizutragen, ist es deshalb weiterhin nötig, auf breiter Basis Versuche anzustellen, nicht zuletzt auch darum, um nach und nach in die Wirkungsprinzipien und -mechanismen einzudringen.

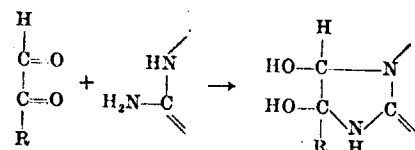
Nachfolgend soll über Versuche an Influenzaviren mit einer bestimmten Stoffgruppe berichtet werden, die, zumindest vom theoretischen Standpunkt aus betrachtet, sehr interessant ist.

Amerikanische Autoren konnten 1957 berichten, daß Glyoxale, das sind bestimmte, sehr reaktionsfähige chemische Verbindungen, die Vermehrung von Influenza- und Newcastle-Disease-Viren in embryonierten Eiern inhibieren können. Auch viruzide, d.h. virusinaktivierende Eigenschaften wurden später festgestellt. Diese Wirkung wird bei anderen virusaktiven Substanzklassen nur sehr selten gefunden, und man nahm deshalb eine direkte Reaktion der Glyoxale mit dem Virus an.

Um diese Annahme der direkten Wirkung auf das Virus zu erhärten, untersuchte Stachelin (1959) konsequenterweise die Reaktion von Glyoxalen mit Nukleotiden, die ja die monomeren Spaltstücke der Nukleinsäuren darstellen, denn bekanntlich ist die Infektiosität des Virus an die Virusnukleinsäure gebunden. Die spektrophotometrischen Untersuchungen dieser Reaktion durch Stachelin machten es sehr wahrscheinlich, daß die Glyoxale spezifisch mit den Guaninbasen der Ribonukleinsäure unter Ausbildung von stabilen Fünfringstrukturen reagieren, wie dies mit Formelgruppe I schematisch angedeutet ist.

<sup>1)</sup> Vortrag, gehalten auf dem Symposium über Desinfektionsmittel und Antiseptika am 24. und 25. September 1965 in Greifswald.

Später vorgenommene Untersuchungen mit einem radioaktiv markierten Glyoxal sprachen ebenfalls für diese hier skizzierte Inaktivierungsreaktion mit dem Guanin.

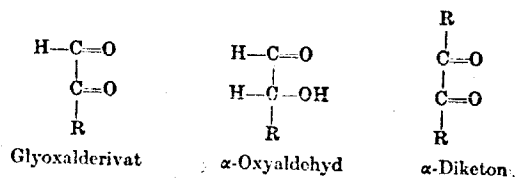


Formelgruppe I

Formelgruppe I

In diesem Zusammenhang ist zu bemerken, daß Guanin eine der 4 möglichen Basen ist, die neben Adenin, Zytosin und Urazil in der Ribonukleinsäure an die Ribose gebunden sind.

Zu Beginn unserer Untersuchungen der Inaktivierung von Influenzaviren prüften wir einige typische Glyoxalverbindungen und verglichen die Wirkung mit dem strukturell verwandten Glyzerinaldehyd, einem  $\alpha$ -Oxyaldehyd, und dem ebenfalls strukturell sehr nahestehenden einfachsten  $\alpha$ -Diketon Diazetyl (s. Formelgruppe II).



Formelgruppe II

Formelgruppe II

Zum Zwecke der Virusinaktivierung wurden zu 0,1 ml virushaltiger Allantoisflüssigkeit 0,9 ml der Substanzlösung hinzugefügt, die 1 mg, nach Möglichkeit in Hanksscher Lösung, enthält. Nach unterschiedlichen Zeiteinwirkungen der Testsubstanzen bei Zimmertemperatur auf das Virus wurde das Material auf  $10^{-3}$  bis  $10^{-6}$  verdünnt. Dann wurden 11 Tage alte Bruteier mit 0,25 ml dieser Verdünnungen beimpft, wobei 6 Eier für jede Verdünnungsstufe benutzt wurden. Wie Kontrollversuche in exembryonierten Eiern zeigten, kam dabei ein Konzentrationsbereich zur Anwendung, der eine Nachwirkung auf das Virus oder eine Schädigung der Wirtszelle ausschloß. Die Bestimmung der  $\text{ID}_{50}$  erfolgte nach Reed und Muench.

Die Abbildung 1 zeigt die Ergebnisse dieser ersten vergleichenden Untersuchungen, wobei wir die beiden oberen Kurven zunächst nicht betrachten wollen. Auf der Abszisse sind die Einwirkungszeiten der Substanzen bei Zimmertemperatur und einer Konzentration von 1000  $\gamma/\text{ml}$  auf das Virus eingetragen.

Auf der Ordinate ist der verbleibende aktive Virusrest aufgetragen, und wir sehen aus dem Verlauf der erhaltenden Inaktivierungskurven, daß die zeitliche Abnahme der  $\text{ID}_{50}$  bei den einzelnen Substanzen sehr unterschiedlich ist. Das Phenylglyoxalhydrat zeigte sich hier am aktivsten, denn schon nach 15 Minuten war der verbleibende aktive Virusrest auf 1 Millionstel der ursprünglichen Menge zusammengeschrumpft. Abgestuft schwächer wirksam waren 4-Biphenylglyoxalbisulfit und Glyoxalbisulfit, was im Lichte der eingangs erläuterten Inaktivierungsreaktion verständlich erscheint. In den Bisulfitverbindungen ist nämlich die reaktionsfähige Aldehydgruppe durch  $\text{NaHSO}_3$  belegt und damit vor Oxydation und Polymerisation geschützt, was aber die Geschwindigkeit der Additionsreaktion an das Guanin negativ beeinträchtigen dürfte. Außerdem muß hier berücksichtigt werden, daß in diesen bei-

den Substanzen das selbst unwirksame  $\text{NaHSO}_3$  gebunden enthalten ist. Dies setzt die Konzentration an eigentlich wirksamer Substanz herab, da immer 1000  $\gamma/\text{ml}$  auf das Virus einwirkten.

Besonders interessant war für uns die in Abbildung 1 strichpunktiert eingezeichnete relativ gute Wirkung des 1,2-Diketons Diazetyl bei guter Zellverträglichkeit, da wir die  $\alpha$ -Diketone erstmalig in solche Untersuchungen einbezogen. Im Gegensatz dazu ist das 1,3-Diketon Azetylazeton nahezu unwirksam. Dies zeigt, daß zur Virusinaktivierung die Nachbarstellung der beiden Carbonylgruppen notwendig ist, denn im Azetylazeton sind die beiden Carbonylgruppen durch eine Methylenbrücke getrennt. Der Glyzerinaldehyd als  $\alpha$ -Oxyaldehyd verhält sich in seiner Wirkung im wesentlichen ähnlich wie das Diazetyl. Die schlechte Wirkung des polymeren Glyoxals erklärt sich aus der Absättigung der aktiven Aldehydgruppen in der polymeren Molekel.

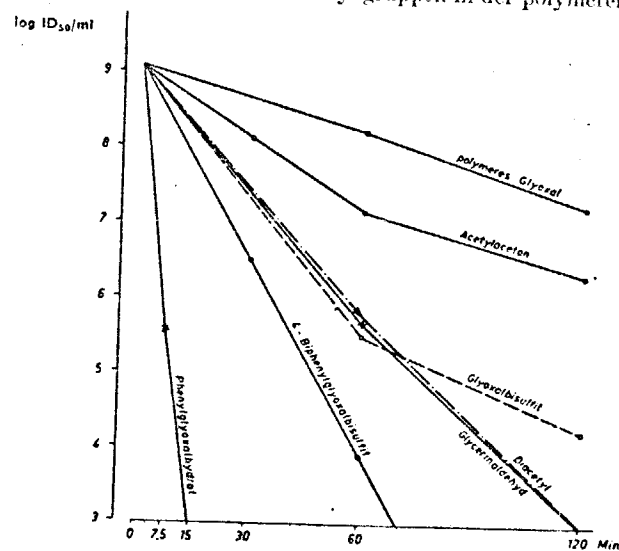


Abb. 1.

Bevor wir zur In-vitro-Austestung von weiteren  $\alpha$ -Diketonen kommen, gestatten Sie mir einige Zwischenbemerkungen. Als wir unsere Untersuchungen auf eine größere Anzahl von  $\alpha$ -Diketonen auszudehnen begannen, hatten wir von Anfang an die Hoffnung, hier auch Stoffe aufzufinden, die nicht nur in vitro wirksam sind, sondern daß man mit ihnen vielleicht auch im Tierversuch therapeutische Erfolge erzielen könne. Die in vitro hochwirksamen Glyoxale erwiesen sich nämlich leider in vivo als wirkungslos mit Ausnahme einiger Biphenylglyoxale, einer Stoffgruppe, die von den italienischen Forschern um Magrassi und Cavallini intensiv bearbeitet wird. Zur Wirkungslosigkeit der Glyoxale im Tierversuch trägt vielleicht die hohe chemische Reaktionsfähigkeit bzw. Labilität, d.h. die leichte Oxydierbarkeit und Anlagerungsfähigkeit, erheblich bei. Die wesentlich stabileren  $\alpha$ -Diketone zeigten in der Tat im Sinne unserer Vorstellungen bei den vorgenommenen Versuchen zwar nicht generell, aber bei einzelnen Substanzen an influenzavirus-infizierten Mäusen reproduzierbare leichte therapeutische Erfolge. Die Tierversuche haben gegenüber der In-vitro-Antestung u.a. den großen Vorteil, daß man auch die unlöslichen Substanzen ausprobieren kann.

Aufbauend auf dem Ergebnis, daß Diazetyl virusaktiv ist, untersuchten wir weitere uns zugängliche lösliche bzw. löslich zu machende  $\alpha$ -Diketone im In-vitro-Versuch. Einige Ergebnisse sind in der Abbildung 2 aufgetragen. Zum Vergleich ist das Phenylglyoxalhydrat noch einmal gestrichelt eingezeichnet, jene Substanz, die nach Abbildung 1 die stärkste Wirkung hatte. Es zeigte sich auch hier, daß die Stärke der Wirkung strukturabhängig ist. Als sehr aktiv erwiesen sich das Zylohexanhexon und das 1.2-Naphthochinon (auch  $\beta$ -Naphthochinon genannt), die selbst das Phenylglyoxalhydrat übertrafen. Während das Zylohexanhexon verhältnismäßig gut wasserlöslich ist, die Zellatmung im Warburgversuch kaum beeinflusst und im Tierversuch auch in hohen Dosen gut vertragen wird, erweist sich das 1.2-Naphthochinon im Warburg- und im Tierversuch als beträchtlich zelltoxische Substanz, die außerdem infolge schlechter Wasserlöslichkeit in 30%igem Methanol gelöst zur Einwirkung auf das

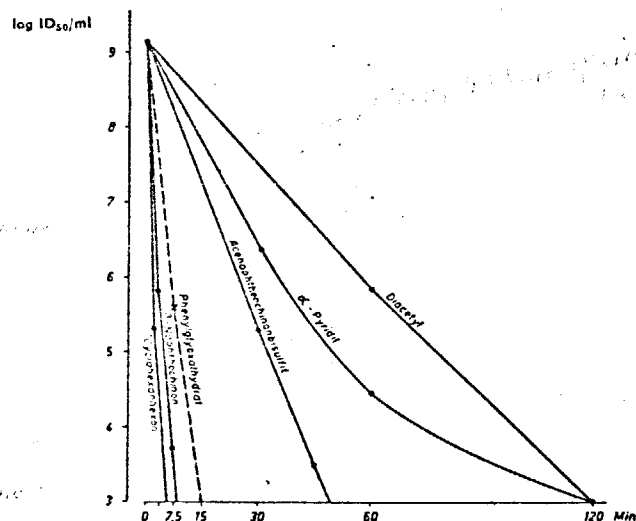


Abb. 2.

Virus gebracht werden mußte. Kontrolluntersuchungen zeigten, daß 30%iges Methanol allein unter den gleichen Versuchsbedingungen praktisch ohne Wirkung ist. Die Überführung des 1.2-Naphthochinons in die wasserlösliche Form durch Einführung einer Sulfosäuregruppe führte zum fast vollständigen Verlust der Wirksamkeit.

Die Ausprüfung dieser nun durch die Sulfonierung verträglicher gewordenen Substanz im Tierversuch steht noch bevor.

Die beiden  $\alpha$ -Diketone Acenaphthenchinon und Phenanthrenchinon sind sehr schwer löslich und in dieser Form nicht ausprüfbar. In diesen beiden Fällen ist es aber möglich, die gut wasserlöslichen Bisulfitverbindungen herzustellen. Wie aus dem Kurvenverlauf des Acenaphthenchinonbisulfits hervorgeht, ist die Wirkung recht beträchtlich. Die Phenanthrenchinonbisulfitverbindung verhält sich offenbar ähnlich, jedoch ist hier eine exakte Ausprüfung deshalb nicht möglich, da die Bisulfitverbindung in der wässrigen Lösung sehr rasch in die Komponenten zerfällt, wobei es zur Ausfällung des unlöslichen Phenanthrenchinons kommt.

Für die Prüfung im Tierversuch ist die Schwerlöslichkeit kein Hindernis, und wir konnten mit dem Acenaphthenchinon in dieser heute betrachteten Stoffgruppe die bisher besten therapeutischen Ergebnisse erzielen.

Das heterozyklische  $\alpha$ -Diketon- $\alpha$ -Pyridil zeigt in vitro eine noch beachtliche Wirkung, jedoch mußte hier Methanol als Lösungsvermittler benutzt werden. Das ebenfalls heterozyklische Fural ist sehr schwer löslich und deshalb auf diese Weise nicht exakt ausprüfbar. Mit rein aromatischen  $\alpha$ -Diketonen konnten wir in vitro bisher keine bemerkenswerten Wirkungen erzielen.

Bei Versuchen an virusinfizierten Mäusen zeigten sich weiterhin Zylohexanhexon und Diazetyl als wirksam. Die entsprechenden Kurven der in vitro erzielten Ergebnisse liegen hier in der Abbildung 2 extrem auseinander, und bei unbefangenen Betrachtungen müßte man erwarten, daß das Zylohexanhexon auch im Tierversuch überlegen sein wird. Dies ist nicht der Fall. Beide Substanzen ergaben praktisch immer den gleichen therapeutischen Effekt. Da die Tierversuche noch nicht abgeschlossen sind, verzichten wir hier auf die Wiedergabe der Einzelheiten des Versuchsablaufes.

Die hohe In-vitro-Aktivität des Zylohexanhexons, die bei auffallend guter Zellverträglichkeit erfolgt, kann man sich aus dem strukturellen Aufbau ableiten. Hier ist der ganze hydroaromatische Ring mit Ketongruppen besetzt, es liegt also eine Anhäufung von wirksamen Prinzipien vor. Aus diesem Grunde haben wir diese etwas kuriose Substanz in unsere Untersuchungen einbezogen und fanden unsere Erwartungen, die wir aus der Formel ableiteten, bestätigt.

Die hier vorgetragenen Untersuchungen haben gezeigt, daß außer Glyoxalen auch die diesbezüglich bisher noch nicht untersuchten  $\alpha$ -Diketone Influenzaviren inaktivieren können. Die  $\alpha$ -Diketone sind außerdem in der Regel viel beständiger und zellverträglicher, und es lassen sich mit einigen Vertretern dieser Gruppe bei Influenzavirusinfizierten Mäusen gewisse therapeutische Effekte erzielen. Darüber hinaus könnten einige dieser Substanzen auch zur praktischen Anwendung als virusinaktivierende Agenzien von Interesse sein, z. B. zur Vakzinherstellung.

Vorfasser: Dr. Horst Mücke und Prof. Dr. Martin Spröbig, 50 Erfurt, Nordhäuser Straße 74.

#### Zur Diskussion sprachen:

##### Herr Profft:

Ich finde die vorgetragenen Ergebnisse sehr interessant. Wie sieht es mit Glyzerinaldehyd bei seiner bekannten Wirkung gegen Mäuseazites aus?

Ich möchte noch anregen, das Fluorenol einzubeziehen, da Fluorenolabkömmlinge bekanntlich zum Teil gute pharmakologische Wirkung haben.

##### Antwort:

Der in der letzten Zeit durch die Versuche zur sogenannten Mehrschritt-Chemotherapie des Krebses interessant gewordene Glyzerinaldehyd zeigte, wie bereits dargelegt, im In-vitro-Versuch etwa die Wirkung des Diazetyls, also eine recht gute virusinaktivierende Wirkung. Leider ergaben sich bei den bisher von uns vorgenommenen Mäuseversuchen keine Anzeichen einer Beeinflussung des Verlaufs der Influenzavirusinfektion durch diesen Stoff.

##### Herr Mach:

Wirken Ihrer Meinung nach die geprüften Substanzen nur auf das Guanin der Virusnukleinsäuren oder während der Replikation des Virus auf den Nukleinsäurestoffwechsel der Wirtszelle?

##### Antwort:

Die Wirkung dieser Substanzen dürfte vorwiegend auf die Virusnukleinsäure gerichtet sein, denn wir fanden bei einigen hochaktiven Stoffen praktisch keine oder nur eine sehr geringfügige Beeinflussung der Zellatmung der Wirtszellen.

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Herr Häussler:

Als leicht zugängliche aliphatische Diketone sollten Zuckerosone in die Prüfung einbezogen werden.

Herr Menzel:

Ausgehend von der Tatsache, daß der Virushemmeffekt in vivo von den zeitlichen Verhältnissen zwischen Infektion und Therapiebeginn deutlich beeinflußt wird, möchte ich die Frage stellen, wann mit der Therapie begonnen wurde?

Herr Mücke:

Optimal ist die prophylaktische oder gleichzeitige Gabe. Der Therapiebeginn ist 3-4 Stunden nach der Infektion noch möglich.



J. Bacteriol. 68:622-6 (1954)  
 COMPARATIVE STUDY OF THE ANTIBACTERIAL PROPERTIES OF  
 ASCORBIC ACID AND REDUCTOGENIC COMPOUNDS<sup>1</sup>

QUENTIN N. MYRVIK AND WESLEY A. VOLK

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Received for publication May 21, 1951

Ascorbic acid has an antibacterial effect against tubercle bacilli (Boissevain and Spillane, 1937; Leitner, 1937; Sirsi, 1952) and many other microorganisms (von Gagy, 1936; Grooten and Bezssonoff, 1935; Lwoff and Morel, 1942a; Ehrismann, 1942). The mechanism of action and the chemical group responsible for the bacterial inhibition are not known.

Bactericidal effect due to a lowering of the pH has been suggested by von Gagy (1936) and Ehrismann (1942); however, Slade and Knox (1950) have found ascorbic acid to be bacteriostatic for a group A hemolytic streptococcus even though the pH was near neutrality. Ehrismann (1942) found that anaerobes were generally stimulated by ascorbic acid, whereas strict aerobes were generally inhibited, suggesting that in the latter inhibition was due to a reduction in the O/R potential of the medium. Lwoff and Morel (1942b) found that inhibition of *Proteus vulgaris* by ascorbic acid was counteracted by the presence of reducing agents and by substances which catalyzed the breakdown of hydrogen peroxide. Similar results were obtained with *Escherichia coli* and *Bacillus subtilis*. They concluded that the inhibition was due to hydrogen peroxide formed during the auto-oxidation of ascorbic acid.

Myrvik *et al.* (1951) reported that some auto-oxidized derivative of ascorbic acid was most likely the active principle against mycobacteria. The addition of catalase in the form of rabbit red blood cell lysates failed to influence the inhibition. Furthermore, they were unable to associate any appreciable tuberculostatic activity with any of the classical breakdown products of ascorbic acid such as 2,3-diketogulonic acid, oxalic acid, furfural, and threonic acid. The active principle was heat-stable, water soluble, and sparingly

soluble in 95 per cent alcohol but was insoluble in acetone, ether, petroleum ether, benzene, carbon tetrachloride, and chloroform.

The present investigation was an attempt to determine the chemical group responsible for the antibacterial properties of ascorbic acid solutions. Evidence is presented which indicates that these inhibitory properties reside in the oxidized enediol (diketone) group.

#### MATERIALS AND METHODS

*Microorganisms used and source.* BCG strain of *Mycobacterium tuberculosis* var. *bovis* and *M. phlei*: Dr. R. S. Weiser, Department of Microbiology, University of Washington, School of Medicine, Seattle, Washington. Ravenel strain of *M. tuberculosis* var. *bovis* and H37Rv strain of *M. tuberculosis* var. *hominis*: Mr. W. Steenken, Jr., Trudeau Laboratory, Trudeau, New York. *Escherichia coli* and *Pseudomonas* sp.: Culture collection, Department of Microbiology, University of Virginia, School of Medicine, Charlottesville, Virginia.

The mycobacteria were maintained at 37 C on Youmans' modification (1916) of Proskauer and Beck's broth. Inocula were prepared by grinding a portion of the surface growth in a sterile mortar and diluting with sterile saline. The other microorganisms were maintained at 37 C in nutrient broth (Difco).

*Antibacterial tests.* The antibacterial tests were conducted using Youmans' (1916) modification of Proskauer and Beck's medium containing 5 per cent Seitz filtered bovine serum. The compounds to be tested were dissolved in distilled water, adjusted to pH 7.0, sterilized by filtration through a Swinny filter, and serially double diluted in one ml amounts of the above medium. Each tube was inoculated with one drop of a bacterial suspension containing approximately  $10^{-2}$  mg of wet weight standardized in a Coleman nephelometer model 9. Cultures of the slow growing mycobacteria were incubated at 37 C

<sup>1</sup> Aided by a medical research grant from the National Tuberculosis Association, through its Medical Section, the American Trudeau Society, and by a research grant, G-3591, from the National Institutes of Health, Public Health Service.

TABLE 1  
Comparative antibacterial spectra of ascorbic acid, reductogenic compounds and alpha diketones\*

Organism	Ascorbic Acid	Inosose	Dihydroxyacetone	Acetoin	Diacetyl	1,2-Cyclohexanedione	1-Phenyl-1,2-Propanedione
H37Rv strain of <i>Mycobacterium tuberculosis</i> ....	150	250	250	250	3	46	21
BCG strain of <i>Mycobacterium tuberculosis</i> .....	150	230	250	250	3	31	24
Ravenel strain of <i>Mycobacterium tuberculosis</i> ...	375	500	500	500	9	125	62
<i>Mycobacterium phlei</i> .....	450	625	625	925	15	125	93
<i>Escherichia coli</i> .....	1,875	2,500	2,500	2,500	31	250	125
<i>Pseudomonas</i> sp.....	2,500	5,000	5,000	2,500	31	500	250

\* Expressed as minimal amount of antibacterial substance in  $\mu\text{g/ml}$  necessary for complete inhibition.

and observed 7 and 14 days after inoculation, whereas cultures of *M. phlei*, *E. coli* and *Pseudomonas* sp. were incubated at 37 C and observed after 24 to 48 hours of incubation. The tube containing the least amount of antibacterial substance and showing absence of growth by visual inspection was the end point.

**Paper chromatography.** Paper chromatography of reductones was carried out and developed according to the procedure of Mapson and Partridge (1949) using butanol-acetic acid-KCN.

**Short term growth experiments.** Nutrient broth (Difco) was dispensed in 5.5 ml amounts in Coleman cuvettes covered with aluminum foil caps. The antibacterial substances were added and the volume brought to 6.0 ml. The tubes were inoculated with 0.1 ml of a culture of *E. coli* which contained approximately  $10^{-2}$  mg wet weight. Nephelometric readings were made at 30 minute intervals in a Coleman nephelometer model 9.

#### RESULTS

The possible role of the enediol group was studied by comparing the antibacterial spectra of ascorbic acid and various other compounds known to give rise through autooxidation to enediol containing compounds. Qualitative tests for the presence of the enediol group were performed by placing small spots of solutions on filter paper, allowing them to dry, and spraying them with 0.04 M 2,6-dichlorophenolindophenol. It was found that seyllo-inosose, dihydroxyacetone, acetoin, diacetyl, fructose, and xylose when allowed to stand at pH 7.0 for several days formed reductones<sup>2</sup> demonstrable with this test.

<sup>2</sup> von Euler and Hasselquist (1959) have reviewed much of their work on reductones and have presented data which suggest the pathway by which some of these reductones are formed.

All the reductogenic compounds tested except fructose and xylose had similar antibacterial spectra (table 1). Diacetyl, which according to von Euler and Hasselquist (1959) undergoes a dimerization reaction resulting in one molecule of reductone and two molecules of acetic acid, was considerably more potent than the other reductogenic compounds even though its antibacterial spectrum was essentially identical to the less potent substances. Fructose and xylose apparently formed insufficient "reductone" to inhibit *M. phlei*, *E. coli*, and *Pseudomonas* sp. and are not included in table 1.

The number of reductones formed from the above compounds was determined by chromatographing one per cent solutions which had been adjusted to pH 9, heated under nitrogen for 5 minutes at 90 C, and adjusted to pH 6. Reductones from dihydroxyacetone, inosose, acetoin, and diacetyl were demonstrated and compared with triose reductone,<sup>3</sup> reductive acid,<sup>3</sup> and ascorbic acid. The comparative Rf values are presented in table 2. Several attempts to chromatograph the reductones arising from xylose and fructose were unsuccessful. The results (table 2) make it seem likely that the reductone arising from dihydroxyacetone is triose reductone. The reductones arising from acetoin and diacetyl appear identical as might be expected from the structural relationships.

The possible antibacterial role of the enediol group was next studied employing 5 per cent ascorbic acid solutions in M/30 phosphate buffer at pH 7.0 and allowing them to autooxidize at 37 C for intervals up to 30 days. Aliquots were removed at intervals for assay against the BCG strain and for chromatographic demonstration of

<sup>3</sup> Obtained from Bios Laboratories, Inc., 17 West 69th Street, New York 23, N. Y.

TABLE 2  
Comparative Rf values of reductones resulting from  
the following compounds

	Rf
Fresh ascorbic acid	0.40
Autooxidized ascorbic acid*	0.56
	0.16
Dihydroxyacetone	0.63
Triose reductone	0.63
Acetoin	0.60
Diacetyl	0.90
Reductic acid	0.70
Inucose*	0.37
	0.19

\* Formed two reductones.

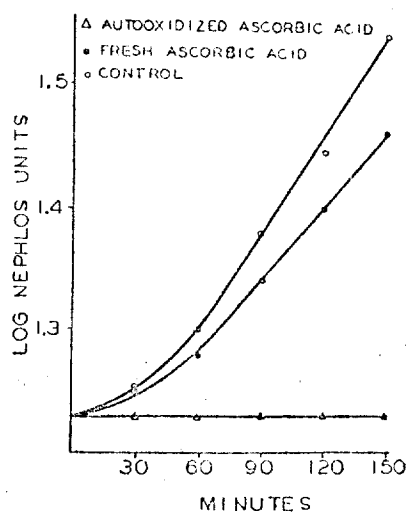


Figure 1. The effect of fresh and autooxidized ascorbic acid on the growth of *Escherichia coli*. The concentration of ascorbic acid was 10 mg/ml in nutrient broth (Difco). Autooxidized ascorbic acid was incubated for 96 hours at 37 C prior to inoculation.

the enediol group. It was noted that the enediol compound corresponding to ascorbic acid disappeared after 2-3 weeks, and two additional unidentified enediol compounds appeared. The last enediol compound to appear persisted long after the 30 days' incubation interval and in one instance was demonstrable for 6 months. The solutions allowed to autooxidize for 30 days were still capable of inhibiting the BCG strain at 100 micrograms per ml in Pechauer and Bock's medium. The persistence of an unidentified

enediol derivative from ascorbic acid suggested that the enediol group might be responsible for the antibacterial effects. Since enediol compounds are oxidized by methylene blue which acts as a mediator for atmospheric oxygen, methylene blue was incorporated in the assay medium in concentration of 1 to 1,000,000. Ascorbic acid was added in varying amounts, and the tubes were inoculated with strain BCG. The addition of methylene blue caused no change in the inhibitory concentration of ascorbic acid, namely, 100 µg/ml, even though no reductones could be detected in the tube containing the most ascorbic acid, namely, 1 mg/ml, using the spot test technique. This was taken as evidence that no enediol compounds remained in the zone which inhibited the growth of the BCG strain, which in turn suggested that the enediol group was not responsible for the antibacterial properties. This idea found further support in short term growth experiments that were conducted in the Coleman nephelometer using *E. coli* as the test organism. Freshly prepared ascorbic acid in a concentration of 10 mg per ml in nutrient broth failed to inhibit the growth of *E. coli*. In contrast, when the same concentration of ascorbic acid was incubated at 37 C in nutrient broth for 96 hours before inoculation, *E. coli* was completely inhibited (figure 1). When as little as 250 µg/ml of freshly prepared diacetyl were incorporated into nutrient broth, *E. coli* was immediately inhibited.

The instability of the enediol group and its direct oxidation to a relatively stable alpha diketone group together with the known antibacterial properties (Bloch *et al.*, 1945; Schalka, 1951) of alpha diketones prompted a study of the antibacterial spectra of alpha diketones. The antibacterial spectra of 1,2-cyclohexanedione and 1-phenyl-1,2-propanedione were demonstrated to be similar to that of ascorbic acid and diacetyl (table 1). These data indicate that the diketone group possesses antibacterial properties that are very similar to ascorbic acid and diacetyl. Qualitative tests for reductones arising from the diketones 1,2-cyclohexanedione and 1-phenyl-1,2-propanedione were negative. Thus, diketones unable to form enediol derivatives nevertheless possess antibacterial properties that are very similar to autooxidized ascorbic acid.

#### DISCUSSION

The mechanism and the exact chemical derivative by which ascorbic acid inhibits bacterial

growth remain to be specifically defined. The data presented support the idea that the alpha diketone group (oxidized enediol) is the responsible chemical group. The similarity between the antibacterial spectra of reductogenic compounds, alpha diketones, and ascorbic acid provides support for this proposal. Furthermore, the short term growth experiments with *B. coli* failed to demonstrate any antibacterial properties of the enediol group but did reveal strong immediate antibacterial effects by the diketone diacetyl and by autooxidized ascorbic acid.

One might expect that 2,3-diketo-gluconic acid would be responsible for the antibacterial effects of ascorbic acid since it is the direct oxidized diketone derivative from ascorbic acid. It has been shown (Penney and Zilva, 1943) that this diketone is very unstable and perhaps could only be inhibitory in a dynamic system. In this regard, Myrvik *et al.* (1952) failed to demonstrate any appreciable tuberculostatic activity with the calcium salt of 2,3-diketo-gluconic acid. Since one of the reductones persisted for six months in a solution of ascorbic acid, it seems more likely that the diketone derivatives which would arise from the unidentified reductones of ascorbic acid might be mainly responsible for the antibacterial properties of ascorbic acid.

Nickerson *et al.* (1953) have reported that low concentrations of reductone (probably triene reductone) have an inhibitory effect on the growth of *E. coli* but stimulate that of *Micrococcus pyogenes* var. *aureus*. They suggest that reductones are inhibitory by binding cobalt into a cobalt-reductone complex. However, data from this laboratory have demonstrated that fresh ascorbic acid is not inhibitory, thus indicating that it is not the enediol group that is responsible for inhibition, but an oxidized derivative of this group. It is well established (Schales, 1951; Bloch *et al.*, 1945) that alpha diketones possess anti-bacterial properties, including marked tuberculostatic activity. Since enediol groups appear to be universally oxidized to alpha diketones, one might expect that in a dynamic system the concentration of alpha diketone derivatives would be sufficient to explain the antibacterial properties of the reductogenic compounds as well as ascorbic acid undergoing autooxidation.

#### SUMMARY

The antibacterial spectra of ascorbic acid, inosose, dihydroxyacetone, acetoin, diacetyl,

1,2-cyclohexanedione and 1-phenyl-1,2-propanedione were found to be similar. Qualitative tests for reductones indicated that inosose, dihydroxyacetone, acetoin, and diacetyl were capable of forming derivatives with enediol groups. In addition, it was demonstrated by chromatography that ascorbic acid undergoing autooxidation yielded two reductones which were unidentified.

Short term growth experiments indicated that the enediol group has no antibacterial properties but that the oxidized enediol (diketone) produces immediate bacteriostasis. It is proposed that the diketone groups resulting from the oxidation of enediol groups are responsible for the antibacterial properties of autooxidized ascorbic acid and the reductogenic compounds.

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## Report on Diacetyl as an Index of Decomposition in Fruit Juices

J. Assoc. Offic. Agr. Chemists 42:294-8, (1954)

By ROBERT C. O'NEILL, Associate Referee (Food and Drug Administration, Department of Health, Education, and Welfare, Atlanta 3, Ga.)

In connection with the keeping properties of the fruit juice concentrates and reconstituted juices, a "rancid problem" has been shown to be due to the buildup of *Lactobacillus* and *Leuconostoc* bacteria which produce diacetyl in the product (1). The Voges-Proskauer colorimetric test is based on the presence of diacetyl and thus may be adapted as a method for its qualitative and quantitative determination (2, 6).

Hill, Wenzel, and Barreto (3), and Beyer (4), have adapted the Voges-Proskauer test to the detection of diacetyl in fruit juices and Beyer has used the adaptation as a sanitary control measure in canning plants.

The principle of the test is as follows: in strongly alkaline solution diacetyl undergoes aldol condensation and combines with guanidine in simple proteins such as creatine. When this complex is coupled with 1-naphthol, a color is formed which can be measured spectrophotometrically (5, 6). At present the method is as follows:

The solution to be tested is a distillate of a fruit juice or the concentrate diluted to the original volume. To 25 ml of this solu-

tion are added 10 ml 5% 1-naphthol in isopropyl alcohol and 4 ml 25% creatine in 49% NaOH, with mixing between additions. The per cent transmittance is read on the spectrophotometer at 550 m $\mu$  (4). A typical calibration curve obtained with a Coleman Jr. spectrophotometer is shown in Fig. 1.

The test also determines another related substance, acetylacetylcarbinol, which recent investigations (4, 7) have shown to occur even more widely in nature, and in larger quantities, in decomposed material.

The method as described has not entirely satisfied its originators, although they find it useful for plant control. In the present investigation the reaction was studied in detail in order to increase its usefulness and reliability. A series of tests on authentic diacetyl in water and in orange juice were so designed that each variable could be studied independently while all other concentrations and conditions remained constant.

Color development depends on time. Figure 2 shows the time-% transmission relationship for 5 ppm diacetyl solution. The curve obtained at other concentrations is of

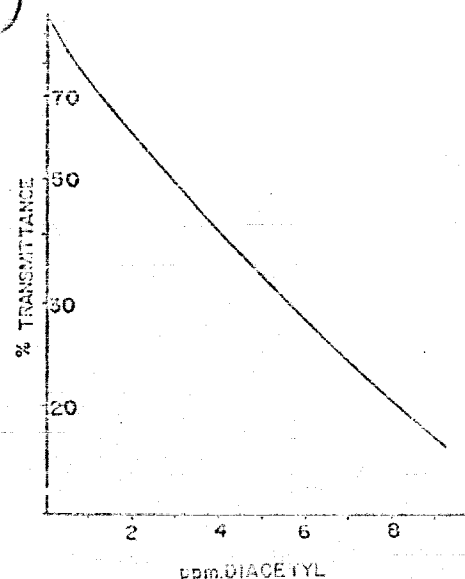


Fig. 1—Calibration curve.

the same shape. The color is stable in the 4-8 minute period after color development begins. Spectrophotometric readings may be taken at any time during this period. Previous methods required reading after 1 minute but Fig. 2 indicates that 4 or 5 minutes is preferable.

The results of varying the concentration of the 1-naphthol in the isopropyl alcohol reagent, using a 5 ppm solution of diacetyl, are shown in Fig. 3. The 1-naphthol concentration is less critical in the flatter part of the curve from 5% up. This indicates that the 5% reagent selected (4, 6) is acceptable.

Figure 4 shows the effect of varying the concentration of NaOH in the alkaline re-

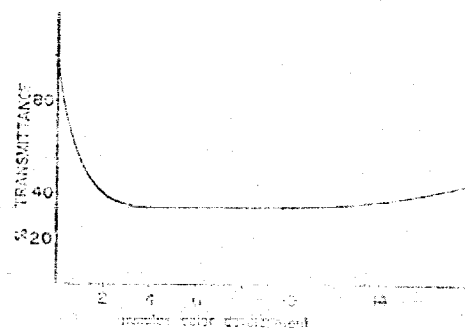


Fig. 2—Time-% Transmittance relationship for 5 ppm diacetyl solution.

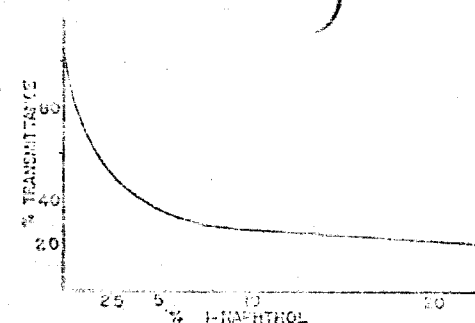


Fig. 3—Effect of 1-naphthol concentration.

agent. The NaOH should always be made up exactly to the specified concentration. The method uses 40 grams per 100 ml, which is in the least critical section of the curve.

Figure 5 shows the effect of varying the concentration of creatine in the alkaline reagent. The concentration of creatine is not critical. However, according to the curve, best results are obtained with 50-80 mg per 4 ml of alkaline reagent.

Variation in temperature has considerable effect on results. The solid line in Fig. 6 shows the effect of developing the color at various temperatures, starting with fresh, cool reagents. The dotted line shows the effect of allowing the test solution to stand at varying temperatures for 6 hours before developing the color at room temperature.

The reaction temperature should be kept below 25°C. The dotted curve would indicate that it might be possible to store the solution at 0°C; however, subsequent experiments have shown that the diacetyl will deteriorate slowly even when frozen. These temperature experiments help explain losses from distillation.

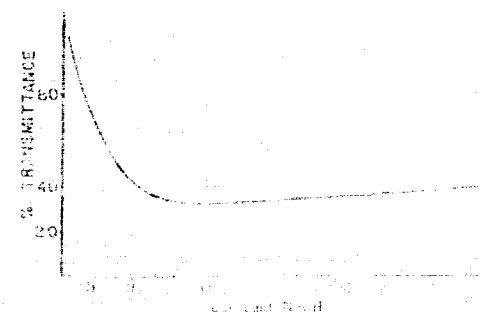


Fig. 4—Effect of varying the concentration of NaOH in the alkaline reagent.

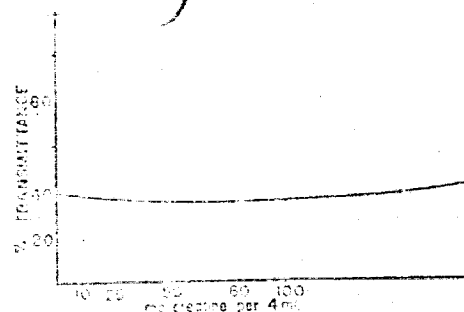


Fig. 5—Effect of orange concentration.

Various amounts of clear filtered orange juice (serum) were substituted for water in making up the 5 ppm diacetyl solution for the color reaction (Table 1). It is seen that orange juice in any concentration interferes with the color reaction in irregular fashion. This test indicates the necessity for separation of diacetyl as by distillation.

Pure citric acid was used instead of orange serum in making the next test. Table 2 shows its effect on the color reaction. Citric acid alone has no effect in the amounts found in ordinary fruit juices.

Table 3 shows the effect on the color reaction if sugar alone is added to the 5 ppm diacetyl solution. It is seen that sugar interferes, but not nearly so much as the orange serum. The diacetyl should be separated from both before determination. Distillation has drawbacks but seems to afford the only practicable separation.

Thus, efforts were made to determine the best kind of distillation to use. Table 4 gives the results of these tests. In all cases 300 ml of original 5 ppm solution was distilled; 50 ml of distillate was collected and diluted back to 300 ml. A 25 ml portion of this was taken for the color reaction.

Table 1. Effect of added orange serum

Orange Serum in Reaction Mixture	% Transmittance of 5 ppm Diacetyl Solution
0	31.5
1	39.5
5	30.5
10	32.1
20	33.0
50	33.6
100	34.6

Table 2. Effect of added citric acid

Citric Acid in Reaction Mixture	% Transmittance of 5 ppm Diacetyl Solution
0	35.0
2	35.0
4	35.0
6	36.0
8	37.5
10	38.5

The simplest all-glass standard taper distilling outfit, with top and neck well lagged, is the best. The loss is 10–15% when 5 ppm diacetyl is present. Complex fractionating systems appear not to be suitable for minute quantities of diacetyl.

Diacetyl in water solution and some of the reagents used in the color reaction deteriorate with age. The following tests were designed to give information about this deterioration. Table 3 shows the length of time each solution stood at room temperature (26°C) before use and the effect on the transmittance, using 5 ppm diacetyl in water. From this table it can be seen that the creatine-NaOH reagent should be made up fresh within a few minutes before use. Diacetyl-water solutions deteriorate appreciably in 12 hours. However, the 1-naphthal in isopropyl alcohol reagent is comparatively stable.

Temperature changes these results somewhat. Under refrigeration the alkaline creatine reagent usually deteriorates more slowly. Loss of diacetyl in water or in orange concentrate continues slowly down to –8°C.

Purity of reagents is not a critical factor

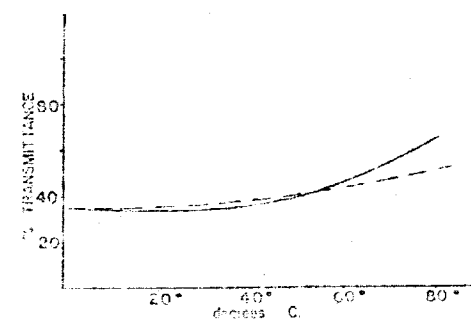


Fig. 6—Effect of temperature. Solid line, cool reagents; color developed at 10–15°C (room temperature). Dashed line, reagents allowed to stand at room temperature; color developed at room temperature.

Table 3. Effect of added sugar

Sugar in Reaction Mixture	% Transmittance of 5 ppm Diacetyl Solution
0	31.5
5	30.0
10	12.0
20	15.5

except in the case of 1-naphthal. An old or impure lot of this substance imparts so much color to the reaction mixture that the range of measurement of diacetyl is restricted.

Almost any fresh diacetyl gives good results in standardization. Old batches should be redistilled and the cut between 87.5 and 89.0°C should be used.

The earlier methods directed the shaking of air into the color reaction mixture. Present tests have shown that it is only necessary to mix the reagents thoroughly and the necessary oxidation proceeds without extra shaking.

A water solution containing 5 ppm diacetyl was chosen for all tests because it represents a median value in the usable range of the color reaction.

It may be concluded at this point that the color reaction as applied to pure diacetyl in water is a dependable analytical procedure. Its isolation by distillation introduces problems that need further study.

Other methods have been used to determine diacetyl. Nickel dimethylglyoxime precipitation (10) gives an accuracy of about the same order as this spectrophotometric

Table 4. Comparison of different types of distillation

Type of Distillation	% Transmittance of 5 ppm Diacetyl Solution
Direct undistilled (control)	31.5
Steam distillation with short-neck still	39.0
Steam distillation with lagged neck still	39.0
Packed column, Raschig rings	19.9
Plate refluxing column (No. 1)	38.5
Plate refluxing column (No. 2)	39.5
Packed column, glass beads	42.5
Simple still (distillation No. 1)	38.0
Simple still (distillation No. 2)	39.0
Simple still (lagged top and neck)	37.5
Simple still (lagged top and neck)	38.5

method when bases in separation of diacetyl by distillation are considered. Its chief drawback has in a small indefiniteness in composition of the red precipitate (11). The precipitation of 2,4-dinitrophenylhydrazine must be carried out in organic solvents, and no suitable means of applying this method to fruit juices was found (8, 9). Diacetyl phosphate separation was studied briefly without finding the conditions necessary to render it quantitative (5).

The above methods all show promise as means of separating the diacetyl from interfering substances such as sugar and ascorbic acid, and especially the other related and concurrently occurring substance, acetyl-methylcarbinol, if this should be found necessary.

When both diacetyl and acetyl-methyl-

Table 5. Effect of aging (Solution I: 5 ppm diacetyl in water; Solution II: 5% 1-naphthal in isopropyl alcohol; Solution III: 2% creatine in 50% NaOH)

Test	Made up Fresh within 30 min.	Stood 1 hr	Stood 8 hr	Stood 24 hr	Stood 60 hr	% Transmittance
1	I, II, III I, II I, II I, II I, II	III	III	III	III	31.5 33.0 38.5 31.5 43.0
2	II, III, I or II, III II, III II, III	I	I	I	I	31.5 33.0 39.0 34.5
3	I, III, II or I, III I, III I, III	II	II	II	II	31.5 33.0 33.0 32.5

Acetaldehyde is present or they frequently are, and an actual resolution of the distillation may show the proportions of each. However, it may not be necessary to separate the two, but seems likely that further tests will show acetyl-methyl-carbinol to be fully as useful as an index of index of decomposition. There may even be an advantage in the fact that acetyl-methyl-carbinol usually occurs in smaller quantities in decomposition than diacetyl, so that the error through loss in separation will be correspondingly lower if the two are determined together.

It is known as acetyl- and acetyl-methyl-carbinol are closely related oxidation-reduction products, proper chemical preparation of these from specimens in oxidative transformation to a common structure might help to improve the usefulness of the test (2, 3).

#### Recommendations

##### For acetyl-methyl-carbinol

(1) That the determination of acetyl-methyl-carbinol be added as a further index of decomposition.

(2) That work be done with a view to improving the adaptation of the Voges-Proskauer reaction to the determination of acetyl-methyl-carbinol, alone and in combination with diacetyl.

\*The journal of *Salmonella* C. and *Shigella* C. Association, see *This Journal*, 42: 25 (1959).

(3) That work on other methods of diacetyl determination be re-examined.

(4) That further experiments to check deterioration rates of diacetyl and acetyl-methyl-carbinol in canned and frozen citrus products be carried out.

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## MILK AND MILK PRODUCTS

[Quality of milk and dairy products from cows suffering from foot-and-mouth disease.] *Kachestvo moloka i molochnykh produktov pri zabolevanii korov yashcheyrom.*

PETKEVICH, L. D. *Shorn. Dold. veses. Soveshch. Moloch. Delu* 1955, pp. 256-65, 8 tables [In Russian] [Timiryazev Acad. Agric., Moscow, U.S.S.R.]

The effect of foot-and-mouth disease on milk and dairy products was studied during an outbreak in 1952 at a farm near Moscow. The infection was marked by a 6-7 fold rise in leucocyte numbers in milk but the lactose-chloride number remained unchanged. Milk yields dropped abruptly by 30-40 per cent, but the butterfat content rose by 70-80 per cent, Ca content increased by about 20 per cent, whilst P content fell somewhat during the first few days after infection. The vitamin content of the milk was also affected; vitamin A and riboflavin declined immediately by about 25 per cent whereas tocopherols, thiamine and ascorbic acid increased by about 80, 20 and 60 per cent respectively. Diacetyl and acetyl-methyl-carbinol production in the milk from diseased cows was drastically reduced, and the coagulation time was increased 3-6 fold. Butter made from pasteurized (85-90°C for 30 min.) sweet cream and the quarg made from the skim-milk were both of high quality.

Some of the butterfat constants were determined, the results showing an increase in the iodine value and in the refractive index and no change in the melting point.

F.L.



# Flavor Retention During Drying<sup>1</sup>

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## Abstract

The influence of spray dryer operating conditions (drying temperatures, extract solids content, nozzle orifice size, and infeed spray pressure) and of variations in the proportions of individual skim-milk components in the extract to be dried, upon flavor retention during drying, was investigated. The influence of those variables upon flavor retention was evaluated by adding either acetoin, acetone, or diacetyl to skim-milk (10 to 50% total solids), drying the skim-milk plus the added flavor under the desired operating conditions, and then colorimetrically analyzing the skim-milk powder for residual amounts of the added flavor compound.

Total solids content of the infeed skim-milk was found to be the major factor influencing the amount of added flavor retained during drying. Diacetyl retention was found to be independent of the particle size of the skim-milk powders. The highest exit air temperatures (about 100 C) and lowest inlet air temperatures (about 160 C) used in this study were found to result in optimum retention of added flavors.

Milk protein extracts retained diacetyl better during drying than lactose solutions of equivalent concentration (48% versus 34% retention). This occurrence may be partially caused by a substantial reduction in the vapor pressure of diacetyl upon addition of milk protein to a diacetyl-water solution. Addition of lactose to a diacetyl-water solution increased the vapor pressure of the diacetyl.

The drying of food extracts is generally accompanied by large losses of volatile flavors. For example, Sivetz and Foote (11) have reported that a spray-dried coffee retains only about 10% of the aromas which were present in the brewed coffee. Extract concentration has

been shown to be the major factor influencing flavor retention during the drying of coffee extracts (11). A high solids (about 50%) extract will lose only a small portion of the volatile flavors present in the concentrated extract.

A correlation between particle size of the atomized extract and flavor retention has been suggested by several investigators (1, 2, 4, 11). They assume that larger particles retain more volatile flavors during drying because the larger particles have less surface area per unit mass (specific surface) available for evaporation of the flavor compounds.

Sivetz and Foote (11) have stated that a low inlet air temperature (less than 204 C) favors the retention of volatile flavors. They suggest that higher inlet air temperatures cause "blossoming" of the drying particles which results in particles with thin walls and a large specific surface.

Exit air temperatures above 80 C were found to produce a stale flavor in spray-dried cheese (3). The stale flavor was attributed to heat damage to the cheese.

A substantial proportion of the previous work has utilized organoleptic evaluation of the dried product to estimate flavor retention during drying. While the ultimate measure of product flavor lies in its organoleptic response, we felt that an objective means of evaluating flavor retention was desirable. In this study, we have analytically measured: 1) the effect of various spray-drying conditions (drying temperatures, extract concentration, nozzle orifice size, and spray pressure) upon the retention of flavor compounds added to skim-milk; 2) the effect of different flavor carriers (selected skim-milk components) upon retention of added flavor compounds; and 3) the effect of storage of the dried product upon retention of added diacetyl. It was also considered of interest to compare the retention of diacetyl added to skim-milk (500 ppm) during roller-, spray-, and freeze-drying processes.

## Experimental Methods

The influence of spray-drying conditions upon the retention of volatile flavors was evaluated by adding 500 ppm of either acetoin, acetone, or diacetyl to skim-milk (10 to 50% TS) or skim-milk constituents, drying the skim-

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milk plus added flavor under the desired operating conditions, and then analyzing the powder for residual amounts of the added flavor compound. Acetoin, acetone, and diacetyl were chosen as test compounds because simple colorimetric tests had been developed for their quantitation in skimmilk systems; they are common food flavors, and they represent a range in volatility and molecular size.

Diacetyl and acetoin were determined by their color reaction with creatine [method of Westerfield (14)]. Acetone was determined by its color reaction with salicylic aldehyde [method of Thin and Robertson (13)].

Particle size of resultant powders was determined by microscopic examination and permeametry. Using a microscope equipped with a stage micrometer, one thousand particles were sized at random. From the data, a weighted mean particle diameter (volume basis) was calculated. The surface average diameter of each powder sample was determined by the method of Pyne (10), using the permeametry apparatus designed by Gooden and Smith (7).

Pilot models of the Coulter spray dryer, Virtis freeze dryer, and double-roller dryer were used in this study.

## Results and Discussion

### OPERATING VARIABLES

**Drying process.** The data in Table 1 show that similar proportions of the added diacetyl (60%) are retained during spray and freeze drying. Substantially greater proportions of diacetyl were retained by spray and freeze drying than by the double-drum drying procedure. The double-drum drying process is similar to vacuum condensing—the moisture is, in effect, boiled from the milk. The vigorous boiling during drying provides little opportunity for formation of a selective membrane at the drying interface, which would preferentially retain the organic volatiles.

**Total solids concentration of infeed skimmilk.** Similar to other investigators, we have

TABLE 1. Per cent retention of diacetyl during roller drying, freeze drying, and spray drying of 30% total solids skimmilk (500 ppm diacetyl).

Trial (no.)	Roller drying      Freeze drying      Spray drying		
	(% retention)		
1	0	59	61 <sup>a</sup>
2	0	67	

<sup>a</sup> Average of ten trials.

found concentration to be the major factor determining the retention of added flavors during drying. The data plotted in Figure 1 show that virtually all of the added diacetyl was retained when 50% total solids skimmilk was used as the flavor carrier. Flavor losses during concentration of food extracts also have to be considered. If the product is concentrated before drying, a large portion of the volatile flavors originally present are distilled from the food product with the water. We found that if diacetyl is added to fluid skimmilk (about 10% total solids) and then spray dried, approximately 35% of the diacetyl is retained in the resultant skimmilk powder. If a batch of the same fluid skimmilk plus diacetyl is condensed in a single-pan batch evaporator to 40% total solids and then spray dried, only 15% of the added diacetyl is retained in the resultant skimmilk powder. Concentration of the skimmilk before drying resulted in greater losses of the added diacetyl than if the skimmilk had not been pre-concentrated. Often there is an optimum concentration at which a food product is spray dried. Coffee, for example, is spray dried at 40 to 45% total solids (11).

**Particle size of atomized extract.** Diacetyl retention was found to be independent of the particle size of resultant skimmilk powders. The mean particle sizes of powder samples were varied by changing nozzle orifice size (0.406 mm, 0.508 mm, 0.635 mm, 0.742 mm) while corresponding changes in infeed spray pressure were made (211 to 106 kg/cm<sup>2</sup>) to

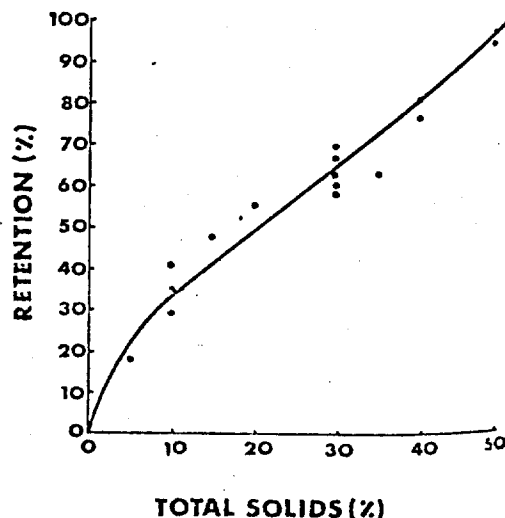


FIG. 1. Influence of infeed total solids on the per cent retention of diacetyl during spray drying of skimmilk. Inlet air temperature, 205 C; exit air temperature, 82 C.

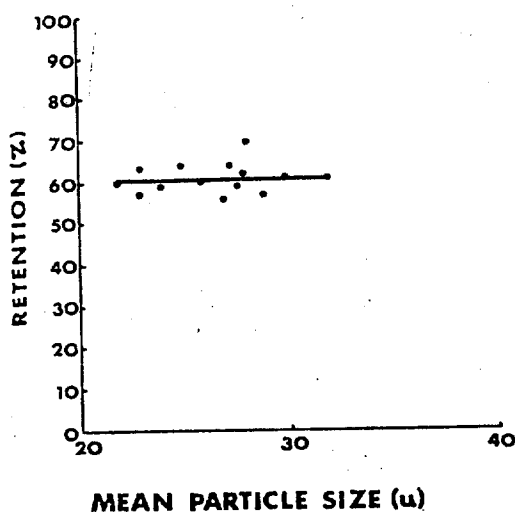


FIG. 2. Influence of particle size on the per cent retention of diacetyl during spray drying of 30% TS skimmilk. Inlet air temperature, 205 C; exit air temperature, 82 C.

maintain a constant infeed rate. As shown in Figure 2, mean particle size (based on mi-

TABLE 2. Per cent retention of diacetyl during spray drying of aqueous systems of selected milk components.

Milk component	Per cent total solids	
	10	15
	— (% R) —	
Skimmilk	43	48
Sodium caseinate	46	51
Sodium caseinate — lactose <sup>a</sup>	26	39
Lactose monohydrate	....	34

<sup>a</sup> Sodium caseinate:lactose ratio = 1:2.

TABLE 3. Diacetyl concentrations of headspace vapors in equilibrium with aqueous systems of selected milk components containing 50 ppm added diacetyl.

Milk component <sup>a</sup>	Trial no.				Average
	1	2	3	4	
	(ppm diacetyl) <sup>c</sup>				
Water	54	52	55	57	54
Skimmilk powder	42	35	42	....	40
Whey powder	60	59	60	65	61
Sodium caseinate	25	27	27	....	26
Whey protein <sup>b</sup>	27	25	23	....	25
Lactose monohydrate	62	65	62	....	63
Sodium chloride	74	....	....	....	74

<sup>a</sup> Ten per cent total solids in final mixture.

<sup>b</sup> Spray-dried fraction from Sephadex G-25 gel filtration.

<sup>c</sup> Determined by GLC headspace sampling.

microscopic examination) varied from about 23.0 to 32.5  $\mu$  in diameter. Mean particle size of the same samples determined by permeametry varied from 20.0 to 33.0  $\mu$  in diameter. The range in particle size within a sample was found to vary from about 5 to 90  $\mu$ . The smallest size, of course, was determined by stack losses.

Assuming that the theory of Brooks (5) and Thijssen (12) is valid, the fraction of a flavor compound retained during drying should depend upon: 1) rate of flavor loss from the particle surface ( $\text{g}/\text{cm}^2/\text{sec}$ ); 2) drying time until the outer membrane of the atomized particle is of sufficient concentration and dimensions to become selective (sec); 3) surface area (per unit mass) available for evaporation of the flavor compound ( $\text{cm}^2/\text{g}$ ). It appears justified to assume a uniform rate of flavor loss, independent of particle size, until a selective membrane is formed around the drying droplet. The surface area per unit mass, of course, is inversely proportional to the diameter. In the case of pure liquid drops, the drying time is directly proportional to the square of the diameter (9). In the case of drops containing, in addition to the solvent, solids which lower the vapor pressure or in which diffusivity of the evaporating substances changes with concentration of the solids, the drying time is longer than for pure liquid drops, due to the effect of both. A reduction in the vapor pressure differential and a decrease in diffusivity with increase in concentration both operate to increase the drying time of a drop of milk over that of a drop of water of the same size. Also, proportionately more water must diffuse to and evaporate from a unit area of surface in the larger particles. Thus, there may be a delay in the formation

of an effective membrane in the larger drops, so that the increased time for loss of volatiles before formation of an effective membrane offsets the effect on volatile loss of the decreased surface area per unit of mass.

**Drying temperatures.** Inlet and exit air temperatures were found to have an effect upon the retention of added flavors (Fig. 3). The higher the inlet air temperature, the lower was retention of the added flavors. Increased inlet air temperatures also resulted in powders of higher moisture contents, larger mean particle sizes, and lower absolute densities. The lower absolute densities were the result of "ballooning" of the atomized particles during drying, as was shown by microscopic examination of powder samples. Ballooning is assumed to be caused by the vaporization of water within the particle at a more rapid rate than the vapors can diffuse to the surface of the particle (9). This causes an expansion of the particle which may stretch (or alter) the selective membrane formed on the surface of the drying droplet. Stretching may delay formation of a selective membrane which may result in additional flavor losses.

High exit air temperatures (90 to 100 C) favor the retention of added flavors. Higher exit air temperatures also produce powders slightly denser, lower in moisture, and larger in size. Improved retention of organic flavors at higher exit air temperatures may be caused by the lower relative humidity of the drying air which should result in an increased rate of water removal from the atomized particles. This should shorten the drying time until a selective membrane is formed around the atomized particles, thereby improving the retention of volatile flavors.

#### CONSTITUENT VARIABLES

**Concentration of flavor compound.** The concentration of diacetyl added to the skim milk prior to drying was found to influence the proportion of diacetyl retained during drying. Diacetyl was added to 30% TS skim milk at concentrations of 500 and 6,000 ppm. Each lot was spray dried using identical operating conditions. Seventy-five per cent of the added diacetyl was retained in the powder when the lower diacetyl concentration was dried. In comparison, 60% of the diacetyl was retained when the higher concentration was dried.

**Extract composition.** Results indicate that individual skim milk constituents vary in their ability to retain added flavors. The data presented in Table 2 show that samples highest in sodium caseinate retained the most diacetyl

during drying. Samples highest in lactose retained the least diacetyl. This may be because the presence of milk proteins in a water-diacetyl solution greatly reduces the vapor pressure of the diacetyl (Table 3). The presence of lactose or milk salts increases the vapor pressure of the diacetyl.

#### RETENTION OF DIACETYL DURING STORAGE

The retention of diacetyl in skim milk constituents during open-air storage and storage in closed plastic bags was investigated. Diacetyl concentration in NFDM gradually decreased during storage in plastic bags (Fig. 4). This gradual loss of diacetyl from the powder may have been caused by migration of the diacetyl from the NFDM to the air trapped in the storage bag.

Diacetyl concentration in the skim milk constituents remained approximately constant during the first days of open-air storage. However, during the 13- to 21-day storage interval, a rapid loss occurred (Fig. 5). Lactose crys-

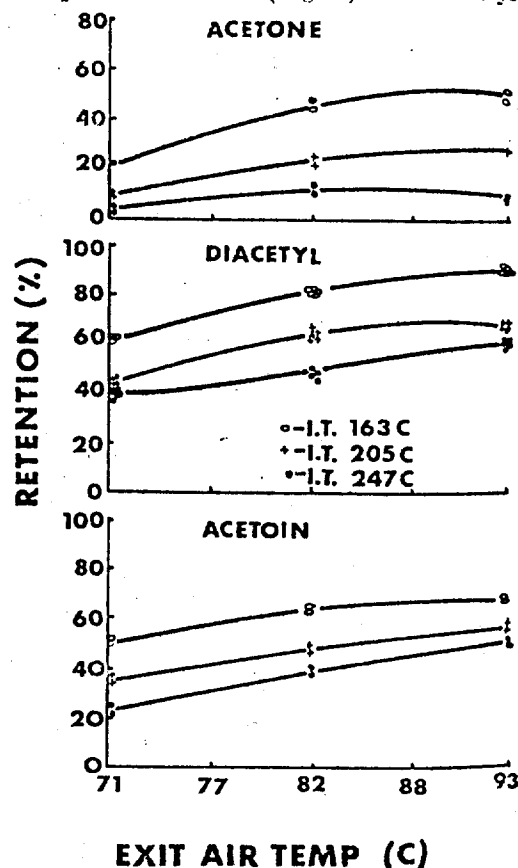


FIG. 3. Influence of inlet and exit air temperatures on the per cent retention (%R) of added volatiles during spray drying of 30% total solids skim milk.

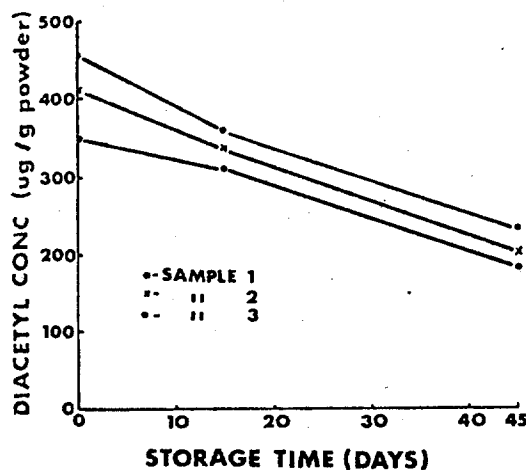


FIG. 4. Retention of diacetyl in NFDM during storage in closed polyethylene bags.

tallization, as evidenced by microscopic examination, also occurred at some time during the 13- to 21-day storage interval. According to King (8), the occurrence of lactose crystallization in NFDM results in the development of a network of fine cracks and interstices along the edges of the lactose crystals. It is possible that a substantial proportion of the diacetyl could diffuse out of the particle through these newly formed pathways.

#### Conclusions

Results of this study suggest that optimum flavor retention during drying is obtained when

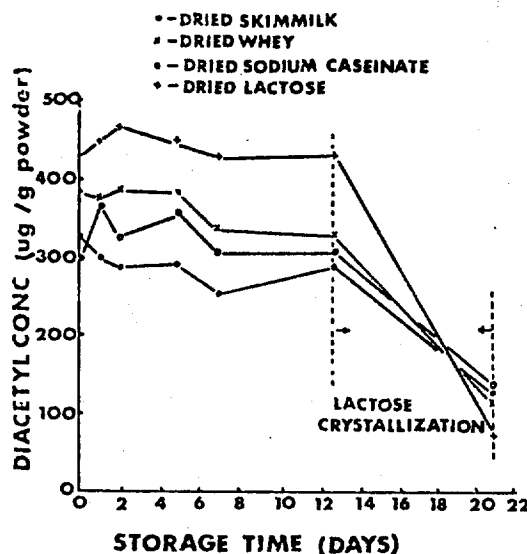


FIG. 5. Loss of diacetyl during open-air storage of selected materials spray dried containing added diacetyl.

a high solids extract (about 50% TS) is dried using a high exit air temperature (about 100 C) and a low inlet air temperature (about 160 C). The retention of flavor compounds added to skimmilk was found to be independent of the particle size of the resultant powders.

When applying these results to natural products, one must consider that a large percentage of flavor compounds may be lost during concentration before drying and that adverse quality characteristics may develop when high exit air temperatures are used. Although a high solids content greatly improves flavor retention during drying, the losses of volatile flavors during concentration may be greater than the savings realized during drying.

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# The Use of Starter Distillate for Flavoring Butter\*

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## SUMMARY

The feasibility of flavoring Canadian butter with starter distillate and with a commercial preparation of synthetic diacetyl was investigated at various concentrations. Although official graders preferred control samples to flavored samples, one-third of the members of a taste panel had a definite preference for the flavored samples and only one-fifth preferred the control samples. The remaining panel members showed no preference. Starter distillate butters were preferred to "commercial diacetyl" butters, and 0.50 oz of distillate per 100 lb of fat was preferred to 0.25 oz or 1 oz, whereas for "commercial diacetyl" the preference decreased with increasing concentration.

The levels of acetyl-methylcarbinol and of diacetyl in the flavored butters were respectively in the range 0.11-0.27 ppm and 0.24-0.64 ppm. These levels did not change even after 1 year storage at -10° F. Peroxide values indicated that the keeping quality of the butter was unaffected by either flavoring.

IT IS WELL ESTABLISHED that butter made from acid cream has poor keeping quality. The requirements for long storage of butter have led to changes in buttermaking procedures to enhance keeping quality. Some of these changes, such as the use of unripened cream, the standardization of the cream to a low acidity, and possibly the washing of the butter granules, have harmed flavor. Since butter is consumed mainly for its flavor, the question arises whether the bland-flavored butter that results from overemphasis on keeping quality would be preferred by most consumers free to choose a more highly flavored butter.

It is desirable to search for means of enhancing butter flavor without harming keeping quality. Butter flavor can be enhanced by ripening the cream, avoiding excessive neutralization of the cream acidity, eliminating the washing of butter granules, adding starter to cream or butter, or adding flavoring materials such as diacetyl or starter distillate. For keeping purposes, ripened cream butter is out of the question. The standardization of the acidity of butter serum to a pH above 7.0 has never been shown to improve keeping quality. Butter washing has been found (White *et al.* 1956) to be of no help to keeping quality under present Canadian conditions. The presence of starter organisms, even when the factor of acidity is eliminated, has been shown by Wiley *et al.* (1939) to harm the keeping quality of butter. However, recent reports by McDowall *et al.* (1960 a, b) indicated that the use of sweet cream starter butter does not harm the keeping quality of butter. The use of pure diacetyl has been found to give an unnatural aroma to butter (Babel and Hammer, 1944; Hunziker, 1940; McDowall, 1953; Mohr and Eichstadt, 1935).

Ruehe and Ramsey (1935) improved the aroma of butter by adding starter distillate to either cream or

butter. Beck *et al.* (1938) found that this procedure does not affect the keeping quality of butter. Various workers (Babel and Hammer, 1944; McDowall *et al.*, 1960 a, b) have reported that the diacetyl content of butter decreased on storage. No pro-oxidative effect of diacetyl in butter has ever been reported at concentrations below 2 ppm.

Since the manufacture of starter butter requires specialized knowledge, skill, and equipment, it was thought preferable to study more practical means of enhancing butter flavor. The objectives of this study were to determine the stability of some flavor additives in stored butter, their effect on the keeping quality of butter, and whether butter flavored with starter distillate or a commercial diacetyl flavoring would be preferred to conventional butter by a small panel of tasters.

## EXPERIMENTAL

**Manufacturing.** Twenty-seven lots of butter were made from Special Grade cream obtained from the Institute Dairy. They involved three flavoring treatments, three levels of concentration, and three replications. The flavoring treatments were: control, starter distillate, and commercial diacetyl flavoring, and the levels of concentration were 0.25, 0.50, and 1.00 oz per 100 lb of butter. The fat content of the cream was 36%; the acidity, initially 0.11%, was standardized to 0.10% with sodium sesquicarbonate. The cream was pasteurized 10 min at 180° F, cooled to 46° F, and held there until churning. Average churning temperature was 48° F. Just before working, each churning of butter was split into three lots for the following treatments: (a) control, (b) starter distillate added, (c) diacetyl flavoring added. The nine churnings were made at intervals of about three weeks, from May to November, to allow time for the analyses.

The starter distillate and the diacetyl flavoring were both from commercial sources, and both contained 0.11% diacetyl and no measurable quantity of acetyl-methylcarbinol. The diacetyl flavoring did not contain any citric, acetic, or propionic acid. Since the loss of diacetyl in the buttermilk is very high (Mohr and Wellm, 1937), the flavoring was added to the butter at the salting stage. The salt content was standardized at 1.9%, and the moisture content at 16%.

Samples from each split churning were saved for the initial analyses. Some 20 one-pound prints from each split churning were wrapped in parchment paper, boxed, and stored at -10° F until examination.

**Methods of analysis.** Samples from each split churning were scored for flavor initially, at 6 months, and at 12 months, by three government graders who had no knowledge of the experiment. A study of flavor preference was made with an experienced taste panel of 15 people. This panel is regularly used for the appraisal of flavor, odor, or other organoleptic properties of various dairy products. In addition, a detailed procedure of tasting was given to each panelist, and several blank tasting sessions were conducted to train the panel. To reduce the work of the taste panel it was assumed that the three control lots in each replicate were alike in flavor, leaving seven samples for examination in each replicate.

A ranking procedure was used to establish flavor preference. A balanced incomplete block design was used whereby the seven treatments in each of the three replicates were presented to the panel in 35 blocks of 5 units. Each block of samples was ranked

\* Contribution No. 37.

Table 1. Effect of added flavoring on flavor scores of stored butter (3 replications)

Flavoring per 100 lb butter	Storage at --10° F	Control			Starter distillate			Diacetyl flavoring			Totals
		1	2	3	1	2	3	1	2	3	
0.25 oz	Initial	39	39.5	40	39	39	39	39.5	38	38	352
	6 months	39	39	39	39	39	39	39	39	39	351
	1 year	38.5	40	39	39	39.5	39	39	39	38.5	351.5
0.50 oz	Initial	40	40	39	38.5	39	38	37.5	39	38	349
	6 months	39.5	40	39.5	39.5	39.5	39	38.5	39	38	353.5
	1 year	39.5	39	39	39	34.5	39	39	38	38	349
1.00 oz	Initial	39	40	40	37	39	38	38	38	38	347
	6 months	40	40	39	39	39	39	38.5	39	38	351.5
	1 year	39	39	39	39	39	39	39	38	38	348.5
Totals		353.5	356.5	353.5	350	347	349	348	347	344.5	

from 1 to 3 in order of preference for flavor by each member of the taste panel. Each treatment appeared five times in the same block with each other treatment.

Samples from each lot were analyzed for diacetyl and acetyl-methylcarbinol at 0 and 3 days; 1, 2, 3, and 5 weeks; 2 months; and 1 year, according to the method of Prill and Hammer (1938). Assuming 100% purity for reagent grade diacetyl, the diacetyl recovery was 93%. For determination of the combined diacetyl and acetyl-methylcarbinol, 20 ml of 40% ferric chloride were added to 100 g of butter before distillation, and the degassing with nitrogen was omitted. Peroxide values were determined by the method of Lips *et al.* (1943).

## RESULTS AND DISCUSSION

**Flavor score.** The flavor scores given by Canadian official graders in Table 1 and analyzed for significance in Table 2 indicate that the graders preferred the con-

Table 2. Analysis of variance of flavor scores

Source of variation	Degrees of freedom	Mean square
Flavoring	2	5.36 <sup>a</sup>
Control vs flavoring	1	8.68 <sup>a</sup>
Starter dist. vs commercial diacetyl	1	2.05 <sup>b</sup>
Concentration	2	0.53
Linear	1	1.91 <sup>b</sup>
Quadratic	1	0.02
Storage	1	0.70 <sup>b</sup>
Linear	2	0.02
Quadratic	1	1.39 <sup>b</sup>
Flavoring × concentration	4	0.17
Flavoring × storage	4	0.58 <sup>b</sup>
Concentration × storage	4	0.53
Flavoring × concentration × storage	8	0.15
Replication	2	0.53
Error	52	0.22

<sup>a</sup> Significant at  $P = 0.01$ .

<sup>b</sup> Significant at  $P = 0.05$ .

trol samples to the flavored butters, and the starter distillate butters to the diacetyl flavoring butters. As expected, preference was in the inverse order of concentration. The 6-month samples received a higher average score than the fresh or the 1-year samples. The flavor preference is indicative of the trend in Canada for official graders to favor butter with "bland" flavor. McDowall (1953) reported a similar observation with New Zealand butter containing 0.05 ppm diacetyl. The New Zealand graders criticized the butter as harsh and "overfull" in flavor.

**Taste panel.** Table 3 shows the rank totals for the 15 panelists. The over-all analysis of variance of the normal varieties (Fisher and Yates, 1953) with the treatment totals adjusted according to Cochran and

Table 3. Flavor ranking of butters flavored with starter distillate or diacetyl flavoring. (Each value is the sum of 15 judgments by taste panel members)

Block no.	Control	Starter distillate (oz) per 100 lb of fat			Diacetyl flavoring (oz) per 100 lb of fat		
		0.25	0.5	1	0.25	0.5	1
1	27						
2	30						
3	29		29	28			
4	32			31			
5	33	29		24			
6	35			29			
7	35		26	25	30		
8	28						
9	32	28				25	
10	32						
11	31	27	26		26		
12	32		32			32	
13	30		31				
14	30	26			27		
15	31				32	31	
16		29			30	28	
17			27	30			
18				34			
19		25		30		26	
20				30			
21			29	32	30		
22		26	29	35	29		
23			29	32			
24		30		27	32		
25				30		33	
26		28		30	30		
27			27	29	33		
28		30	27			32	31
29			26		28		
30		29				29	36
31					31	27	32
32		30			27		
33		30	26				31
34			28		32	30	
35		27	32		31		
Totals	469	425	424	444	449	454	485
Adjusted totals	457.2	440.6	440.2	447.7	449.6	451.5	463.2

<sup>a</sup> Increasing values = decreasing preference.

Cox (1957) indicates that the difference between treatments is almost significant at  $P = 0.05$ . The adjusted treatment mean square on a unit basis was 2.04, against a  $P = 0.05$  of 2.24. The concordance coefficient (Kendall, 1948) indicated also that the members agreed on a difference between treatments.

Since taste preference is known to vary with individuals, an over-all analysis of variance of preference data could be misleading when there are reverse preferences by substantial portions of the panel. To obtain the full information from the preference data, the members were grouped on the basis of the significance of the chi-square values of their preference. Table 4 indicates that five members preferred the

Table 4: Grouping of panel members according to their flavor preference

Panelists	Treatment						Chi-square
	B	C	D	E	F	G	
Prefering flavored butters	No. times in five the treatment was preferred to the control.						
1	5	4	1	1	2	1	12.1
2	4	5	1	4	5	5	20.4
3	4	3	2	1	5	5	14.0
4	3	5	5	4	3	3	12.4
5	5	5	5	5	4	5	26.8
Prefering control sample							
6	0	1	2	0	2	2	12.1
7	0	0	3	1	3	2	12.4
8	0	1	1	0	1	0	20.4
Showing no preference							
9	3	3	2	1	1	2	4.4
10	2	3	1	1	1	3	6.0
11	1	1	4	3	3	4	7.6
12	3	3	0	4	3	3	7.6
13	3	1	3	0	3	2	7.6
14	3	2	2	2	4	1	4.1
15	1	2	3	4	3	2	4.4

flavored samples to the control, three preferred the control to the flavored samples, and seven showed no preference.

For the panelists showing an over-all preference for the flavored butters, the analysis of variance of the normal variates after adjustments for the treatments for incomplete block analysis (Cochrane and Cox, 1957) showed a highly significant difference between treatments. The multiple range test of Duncan (1955) indicated that the members preferred the flavored butters over the control but had no preference among the flavored butters, varying in flavoring type and in flavoring concentration.

For the three members showing an over-all preference for the control over the flavored samples, the analysis of variance showed a significant difference between the adjusted treatments. The multiple range test of Duncan established that these panelists did not distinguish between the control and the low level of starter distillate, but preferred these to all other treatments. Among the flavored samples, the low and medium levels of starter distillate were preferred to the samples with diacetyl flavoring or with high level of starter distillate.

**Acetylmethylcarbinol.** The average acetylmethylcarbinol content of the control samples was 0.11 ppm. This value is in agreement with the value of 0.16 ppm reported by Mohr and Wellm (1937) for sweet-cream butter. The acetylmethylcarbinol content of the flavorings used was not measurable by the method used. However, the addition of flavorings to butter effected a slight increase in the acetylmethylcarbinol content, more so for commercial diacetyl than for starter distillate. Even at the rate of 1 oz of flavoring per 100 lb of butter the acetylmethylcarbinol content was raised only to 0.17 ppm and 0.21 ppm for starter distillate and for commercial diacetyl, respectively. The variates storage and concentration had no effect on the acetylmethylcarbinol content of the butter.

According to Hedrick and Hammer (1942), starter butter may contain as much as 16 ppm of acetyl-

methylcarbinol. McDowell *et al.* (1960a,b) obtained 1.4-3.9 ppm in their sweet-cream starter butter. They found that storage for 8 months at 17° F effected a slight increase (8-15%), significant at  $P = 0.01$ , in the acetylmethylcarbinol content of the butter. The increase was not significant after 4 months. Presence of starter and relatively high storage temperature may have accounted for this slight increase.

**Diacetyl.** The diacetyl content of the control samples averaged 0.16 ppm. This value is in the range of those reported by other workers for sweet-cream butter. Cox (1942) found 0.02-0.20, average 0.07 ppm. Barnicoat (1937) 0.05 ppm, and Mohr and Wellm (1937) 0.2 ppm.

Starter butters can have as much as 2 ppm diacetyl, as in Danish butter (Pette, 1918), or have less than 0.5 ppm, as in most French butters (Pien *et al.*, 1937). According to Barnicoat (1937) and Davies (1937), the diacetyl content of mild starter butter is about 0.4 ppm, and that of "high-acid" butter is 1.5 ppm. Davies classified butters on the basis of their diacetyl content as follows: 0.2-0.6 ppm is mild, 0.7-1.5 ppm is full, and 2 ppm is strong. With this classification, the flavored butters in our experiment would fall into the category of mild (Tables 5 and 6).

On adding flavoring to butter, there was a loss of about 50% of diacetyl. This percentage loss increased with the level of added flavoring. McDowell (1953) found a similar loss, which he attributed to loss to the atmosphere and to wash water during churning. Since the flavoring was added to the butter at the

Table 5. Diacetyl content (ppm<sup>a</sup>) of stored butter flavored with starter distillate

Storage at -10° F	Control <sup>b</sup>	Oz of added distillate per 100 lb of butter			Mean
		0.25	0.50	1.00	
Fresh	0.14	0.26	0.30	0.42	0.28
3 days	0.15	0.25	0.30	0.43	0.28
1 week	0.16	0.26	0.29	0.44	0.29
2 weeks	0.14	0.20	0.28	0.38	0.25
3 weeks	0.17	0.24	0.31	0.40	0.28
5 weeks	0.18	0.26	0.32	0.42	0.29
8 weeks	0.18	0.26	0.31	0.40	0.29
1 year	0.15	0.24	0.29	0.43	0.28
Mean	0.16	0.25	0.30	0.41	0.28

<sup>a</sup> Individual values are averages of three replicates.

<sup>b</sup> Control values are averages of nine churnings.

Table 6. Diacetyl content (ppm<sup>a</sup>) of stored butter flavored with diacetyl flavoring

Storage at -10° F	Control <sup>b</sup>	Oz of added diacetyl per 100 lb of butter			Mean
		0.25	0.50	1.00	
Fresh	0.14	0.31	0.44	0.51	0.38
3 days	0.15	0.29	0.45	0.50	0.37
1 week	0.16	0.28	0.47	0.54	0.38
2 weeks	0.14	0.27	0.42	0.53	0.37
3 weeks	0.17	0.28	0.44	0.50	0.38
5 weeks	0.18	0.29	0.43	0.58	0.37
8 weeks	0.18	0.29	0.48	0.57	0.39
1 year	0.15	0.26	0.42	0.55	0.34
Mean	0.16	0.29	0.44	0.50	0.37

<sup>a</sup> Individual values are averages of three replicates.

<sup>b</sup> Control values are averages of nine churnings.



saltling stage in our experiment, wash water was not a factor. However, the diacetyl could have disappeared by volatilization and by oxidation or reduction.

Tables 5 and 6 show the diacetyl content of butter for both flavorings at various levels. Table 7 indicates

Table 7. Analysis of variance of diacetyl values

Source of variation	Degrees of freedom	Mean square
Flavoring.....	1	0.437*
Concentration.....	2	1.164*
Storage.....	6	0.002
Flavoring $\times$ concentration.....	2	0.126*
Flavoring $\times$ storage.....	12	0.005
Flavoring $\times$ concentration $\times$ storage.....	12	0.008
Error.....	84	0.075

\* Significant at  $P = 0.01$ , using the appropriate significant interaction to test for flavoring, concentration, and storage.

that in spite of the equivalent strength of the flavorings, more diacetyl was imparted to butter by commercial diacetyl than by starter distillate. This is substantiated by the significant interaction between flavoring and concentration.

Table 7 also shows that storage for 2 months or 1 year at  $-10^{\circ}\text{F}$  did not change the diacetyl content of the butter. In two published papers, McDowall (1960a, b) found that storage of sweet-cream starter butter at  $14^{\circ}\text{F}$  decreased the diacetyl content by 14–20% in 4 months. Analysis shows their data were significant at  $P = 0.01$ . Prolonged storage for 8 months did not change the diacetyl content. The authors did not mention whether the butter was salted. The presence of microorganisms, along with the relatively high storage temperature and possibly a low salt content, probably accounts for the decrease in diacetyl content during storage. As reported by Babel and Hammer (1944), many other workers have observed that the diacetyl content of butter decreased on storage; this was generally attributed to the reducing action of microorganisms or to oxidation by air, especially in the presence of acid or in the absence of added salt. With salted butter, several authors have reported that the diacetyl was more stable.

The keeping quality of the butter was not affected by the presence of flavorings, as indicated by the absence of any difference in peroxide values between the treated and the control samples after 1 year of storage at  $-10^{\circ}\text{F}$ .

#### Acknowledgment

The authors are indebted to the official graders and to the Institute staff for cooperation in grading and ranking the butters.

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Date Sept 19, 1973Chumleas Laboratories Inc.  
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Draft

Gentlemen:

As you know, the Food & Drug Administration is reviewing the safety of all food additives currently on the list of substances Generally Recognized as Safe (GRAS). On each additive an individual monograph is prepared, following a search of the literature published in recognized scientific journals during the past half-century. The monograph author abstracts and presents factual data within a prescribed format, without expressing any opinion of his own. It is his duty to make such information as comprehensive as possible, by such additional inquiries as may be deemed necessary.

I am currently preparing a monograph on "starter distillate" that will include a section on its main component, diacetyl, but not on its other components. I have information suggesting that your Company is or was a supplier of this substance, or rather, mixture, used as a butter flavor intensifier.

There appears to be no formal standard of identity, or specification of "starter distillate" as such, recognized by the FDA, or written into CFR. Based on the published literature I propose to identify "starter distillate" as a steam distillate of starter resulting from the culture of any or all of five species of bacteria together with the medium in which they are cultured, namely, milk or fractions of milk. The five species are Streptococcus lactis, S. cremoris, S. diacetylactis, Leuconostoc citrovorum, and L. dextranicum.

Should you have any comments upon this identification I shall be glad to receive them as soon as possible. I shall also be glad to receive any other information about starter distillate or diacetyl that you may consider relevant to a discussion of its safety as a food additive.

Very truly yours,

Humphrey Sassoon, PhD

- HS:ds

Old Food & Drug

September 17, 1959

*Fischbach*  
*Connelly*  
*Howe*  
1815

MEMORANDUM OF INTERVIEW

PRESENT: Dr. Ralph Hussong, National Dairy Corporation, New York, N. Y.

Mr. E. M. Polak, Polak's Frutal Works, Inc., Amersfoort, Holland  
(AF 19-140)

Mr. L. L. Ramsey (part time) ) Division of Food  
Dr. W. Horwitz ) Food and Drug Administration

Mr. P. J. Schouboe, Division of Pharmacology  
Food and Drug Administration

The visitors came in to determine the status of a starter distillate concentrate, to be used in margarine, with respect to the Food Additives Amendment. The product is apparently made in Holland.

Mr. Polak presented data on the general description, specifications, and levels of use of the starter distillate concentrate (Copy attached). In response to a specific question he stated that citric acid was added in the process.

It was pointed out that starter distillate and diacetyl are allowed for use in margarine as outlined in the Food Standards - Part 45.1 (a) (1) (3) (iv). No mention is made of the allowable concentration of acetyl methyl carbinol.

We pointed out that while diacetyl was an accepted flavor for this use, we were still considering the status of acetyl methyl carbinol. The latter component was stated to be present in starter distillate in a concentration about five times that of diacetyl.

The visitors outlined the general production techniques of their starter distillate concentrate and it appeared that by this method, they were producing what is generally recognized as starter distillate. They pointed out that the acetyl methyl carbinol in the end product, margarine, would be less than that occurring naturally in sour cream butter. The visitors will present figures demonstrating the relative concentrations of these components in various natural and other food products.

They will request a ruling on whether their starter distillate concentrate is a food additive at the time the additional information is submitted.

P. J. Schouboe  
Biochemist

cc: NY  
OC (Attn. Mr. Checchi)  
BPS  
DF  
DP

PJSchouboe:lim  
9-18-59

INIT:Wdh

NOTICE-STARTER DISTILLATE

The Role of  $\beta$ -Carotene in the  
Oxidation of Butter

W. Schuller (From the Daily and Agricultural  
Microbiology Institute at the Vienna College for  
Soil Cultivation - Director: Prof. Dr. A. Zeilinger)

Introduction

Different substances, which have become components of butter as a result of the type of milk treatment used, as well as substances which occur naturally in milk and milk fat, affect the course of autoxidation of butter fat. According to their mode of action, these products are known as pro- or antioxidants. Numerous scientific studies are concerned with the oxidation-inhibiting and promoting behavior of these products. However, the results of research on the oxidative participation (synergistic action) of various vitamins are mutually contradictory.

The task of the present study is to clarify the question as to the manner in which  $\beta$ -carotene takes part in the autoxidation of butter fat.

Status and Formulation of the Problem

The essence of butter oxidation consists in the addition of oxygen to the double bonds of unsaturated fatty acid residues under formation of peroxides, followed by the splitting of fatty acid chains and formation of aldehydes and ketones. The content of fatty acids with carbon atoms linked by double bonds is considered as the primary factor of oxidizability. These fatty acids exhibit a high reactivity as a result of their unsaturation. Thus, they readily undergo oxidation and oxidize other fatty acids present in a sort of chain reaction.

According to Greenbank (7), these catalytic processes are promoted by the

presence of free fatty acids. Among saturated fatty acids, high-molecular acids are more subject to undergo such reactions than low-molecular acids.

Thome (32) and Mattson (20) have found relations between the unsaturated fatty acid content of butter and the length of the oxidation induction period. No direct relations could be established between the degree of oxidation and the percentage content of unsaturated fatty acids.

Schmalfuss (29) noted that fats consisting only of saturated fatty acid residues can also undergo oxidation. The induction period in this case had a corresponding greater length.

Reinart (26) believes that highly unsaturated fatty acids of the C<sub>20</sub> - C<sub>22</sub> series are responsible for the oxidation susceptibility of butter fat.

The oxidation of butter fat by light is based on a kind of photochemical process, which, in case of a quantum absorption of light, initiates the following chain reaction illustrated by Böhmer, and Grossfeld (1):

1.  $F + h\nu \longrightarrow F'$
2.  $F' + O_2 \longrightarrow F'O_2$
3.  $F'O_2 + F \longrightarrow FO_2 + F'$

The fatty acid molecule (F) is activated by irradiated light energy (hv) to oxygen addition (F'O<sub>2</sub>). This stimulated peroxide transmits the reaction energy further and is thus converted into a normal peroxide.

According to Eggert (6), only unsaturated and aromatic compounds are capable of absorbing light in the visible and ultraviolet wave range. Electrons capable of being excited are located on chromophore groups, of which one kind, the ethylene group  $\overset{|}{\text{C}}=\overset{|}{\text{C}}-$ , is the building component of unsaturated fatty acids and also of carotene.

According to Schmalfuss (29), when unsaturated fatty acids are irradiated

with light of a wavelength below  $4100 \text{ \AA}$ , they are definitely susceptible to oxidation.

Conjugated fatty acids are especially subject to accelerated oxidation under the effect of light. Lembke (16) was able to detect and determine conjugated fatty acids in butter.

Pont (23,25) established a taste deterioration due to oxidation in milk samples irradiated with direct sunlight and diffuse daylight.

The oxygen content of butter is also of great importance in its susceptibility to oxidation. In contrast to the previous opinion that dispersed aqueous butter ("butter water") must be considered primarily as an oxygen carrier, studies performed by Loftus Hills (17) have shown that oxygen is soluble in liquid butter fat to the extent of  $2.55 \text{ ml/100g fat}$ . Plastic butter contains up to 55% liquid fat.

Chalmers (3) points out especially the different types of milk treatment (pasteurizing, evaporation) which can modify the oxygen content of milk fat and thus of butter. He also indicates further the great importance of reducing bacteria; as a result of the biological oxygen demand of such bacteria, only a very small amount of oxygen is available to satisfy the chemical oxygen requirements.

Aqueous butter ("butter water") can play an effective role in the course of oxidation, in view of its function as an oxygen carrier and also its possible content of heavy metal traces and different salts acting as pro-oxidants.

Greenbank (7) showed that butter with a low liquid content tends to form aldehydes and butter with a higher liquid content tends to form acids.

The oxidation promoting action of sodium and magnesium chloride was established by Loftus Hills (17). As a result of contact with milk and milk

fat, the content of metal ions can increase above the normal amount.

Traces of heavy metals are of special significance. Among all metals, copper is considered as the strongest transmitter of oxygen. According to Chalmers (3), copper ions deposited around a fat droplet or dispersed in butter act as a catalyst by activating oxygen. Similar observations were made by Tollenaar (34), Greenbank (7), Pont (24) and Krykowsky (14).

Natural components can play a decisive part in the course of oxidation as oxidation regulators or catalysts. Among such components, phospholipids can be mentioned first; their content in butter is equal to 0.02%. They surround fat droplets, according to King (11), as a closed layer, so that their fatty acid fractions extend into the fat phase and the hydrophilic OH groups of phosphoric acid are directed against the surrounding aqueous phase. From this arrangement, we can conclude that phospholipids act as transmitters of oxygen from the aqueous phase into the fat phase. According to Greenbank (7), they are also readily oxidized in view of their content of highly unsaturated fatty acids.

Lea (15) also believes that phospholipids play an important role as oxidation promoters of milk fat, although they are able to affect oxidation process in a significant way only as the so-called lipoprotein complex. In the dispersed state, phospholipids are inactive; in view of their low content in butter, they have practically no effect on highly unsaturated fatty acids. When combined with tocopherol, an oxidation-inhibiting action of phospholipids could even be established.

Tocopherol itself is considered as an antioxidant; its oxidation-inhibiting action was shown, among others, by Schulte and Schillinger (30) in their tests on the oxidation of vitamin A and carotene.



According to Reinart (28), tocopherol exerts an oxidation inhibiting effect; this effect is hardly noticeable in butter, however, in view of its low concentration (2.5 micrograms/100g).

Krukowsky et al. found that milk fat was made resistant to oxidation by 2.614 micrograms of tocopherol per 100g fat, in spite of the addition of 1mg Cu/liter.

When the butter manufacturing batch is acidified, diacetyl is formed by certain aroma-producing bacteria, it is a diketone, which is very easily reduced and for this reason is capable of oxidizing its environment.

Along with an oxidative attack on butter, Reinart (27) also found that such an effect is exerted on butter dyes such as carotene and bixin.

According to King (12), diacetyl reacts as a hydrogen acceptor. Water acts as a hydrogen donor, while unsaturated fatty acids and butter dyes take up the oxygen.

Gyllenberg (8) studies the effect of vitamin C in milk products. According to the results obtained, the reason for the readiness, on the part of vitamin C, to inhibit oxidation probably lies in its easy oxidizability to dehydroascorbic acid. Results obtained by Greenbank (7) agree with this finding. Ascorbic acid especially affords protection against oxidation caused by metal contamination.

Krukowsky et al. (14) provided valuable insight into the mode of oxidation of ascorbic acid to dehydroascorbic acid and its interplay with the oxidation of fat. Only a definite quantitative ratio between ascorbic acid and dissolved oxygen was capable of preventing the formation of an oxidation flavor.

The oxidation behavior of vitamin A is of great interest. Special attention, however, was given to the effect of  $\beta$ -carotene.

Thompson and Steenbock (33) performed tests on the combined (coupled) oxidation of carotene and fat in lard, olive oil, cottonseed oil and soybean oil. A shortening of the induction period by 20 - 40 hours was found in carotene-containing samples, as compared to reference samples.

Reinart (26) found that carotene has an antitoxidant effect up to the 10th day of the test, and from this time on exerts a pro-oxidant effect.

Schulte and Schillinger (30), while testing the behavior of vitamin A acetate and carotene in fat oxidation, found that fat had a higher oxygen consumption in the presence of vitamin A and carotene.

Holmann (10) studied photometrically the course of oxidation of methyl linoleate and the simultaneous breakdown of carotene, and was able to establish that almost all of the carotene is broken down before 10% of the ester is oxidized.

Diemayr et al. (5) also performed their respective studies by using esters as models. They found that the peroxide values of the carotene-containing samples increased by 400%, as compared to carotene, along with other dyes, and found a higher oxygen uptake when carotene was present.

Chevalier et al. (4) found that carotene behaves as an antioxidant in the dark and as a pro-oxidant under the effect of light.

According to Chalmers (3), carotene exerts an oxidation-inhibiting effect during the deterioration of fat.

The above, partly contradictory opinions, obtained by means of different and perhaps not always fortunately selected methods and processes, prompted us to undertake the studies described in this article.

Tests were to be carried out with pure butter fat, and the amounts of added carotene were not to exceed the natural concentration found in butter. Finally, a sensitive method of peroxide measurement was expected to help

clarify this question.

#### Method

##### A) Preparation of a Carotene-Free Butter

Thus, it was only possible to treat fresh butter with oxidation-neutral agents as sparing as possible in such a way that this treatment would comply with the necessary requirements. A purely chemical removal of carotene can be achieved only by saponification of the fat. A reconversion of the soaps to butter fat would not be justified for reasons of fat protection (preservation). However, it was possible, according to Böhmer and Grossfeld (2), to bind carotene by adsorption on surface-active compounds. This method is often used for the purification of fat, and is accompanied by the removal of phospholipids and tocopherol. This method appears in principle to be suitable for the present purpose.

The original butter was prepared from fresh sweet cream containing 30% fat in a glass Alfa-drop butter barrel. The design of the barrel prevents the butter manufacturing batch from coming into contact with metal parts. In order to allow the evacuation of the butter barrel and the blowing in (injection) of nitrogen, a device for adding products to the barrel was installed. Further, in order to afford protection against light, the glass parts were coated black.

The butter was made at a temperature of 14° C until the grain assumed a pea-size dimension. After the buttermilk was drawn off, the butter grain was washed with fresh tap water until the wash waters were completely clear, immediately mixed with 5% pulverized active carbon ("medicinal grade") in a closed glass vessel and heated in a water bath to 95° C. The butter-active carbon mixture was held at this temperature under frequent stirring and shaking. Since a separation of this mixture by filtration soon proved to be impossible in

view of the relatively high content of active carbon and also high losses of fat, an attempt was made to achieve a rough preliminary separation of butter fat and active carbon in a centrifuge. A Gerber milk centrifuge of about 1000 rpm was available; test tubes with a 100 cm<sup>3</sup> content were used as centrifuge tubes, and an appropriately fitting holder was manufactured. An electric heating coil mounted on the centrifuge made it possible to maintain a working temperature of 35°C required for this purpose. In this manner, it was possible to achieve a rough separation of active carbon and butter fat after a centrifuging time of 20 minutes. Subsequent fine separation was done in a double-jacket hot water funnel by means of a Schleicher & Schüll No. 588 folded filter.

Carotene ( $\beta$ -carotene, crystalline, synthetic, Hoffmann-La Roche) was added to the fat directly from a freshly broken vial.

The accurately weighed amount of fat was emulsified in a Bauknecht mixing apparatus (Fig. 1), equipped with a device for evacuation and injection of nitrogen, with the calculated amount of distilled water. This process was carried out in a continuous stream of nitrogen at 40 C. In order to prevent the separation of the emulsion into a fat and water phase during cooling, a special kneading apparatus (Fig. 2) was used, which made it possible to carry out the kneading process under a nitrogen atmosphere. This kneader was made from plexiglass in such a way that no metal part came into contact with the butter. In this apparatus, the fat-water emulsion was solidified under continuous kneading in a nitrogen atmosphere, then expelled, broken up into portions with a plexiglass spatula, packed in parchment paper, and thus exposed to autoxidation.

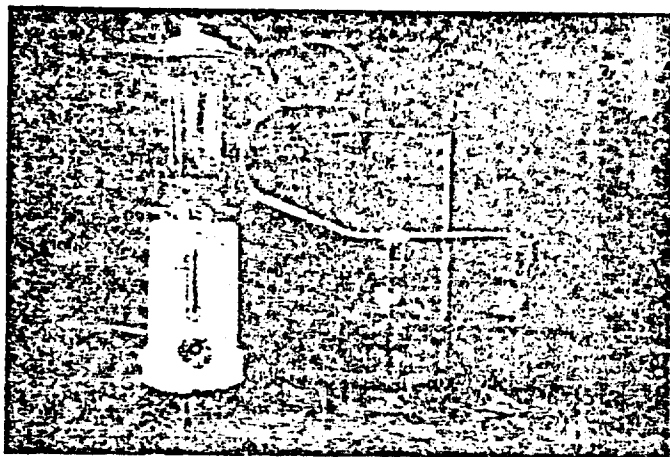


Fig. 1

- a) Washing flasks with pyrogallon and caustic potash for nitrogen purification;  
b) apparatus for the preparation of the emulsion.

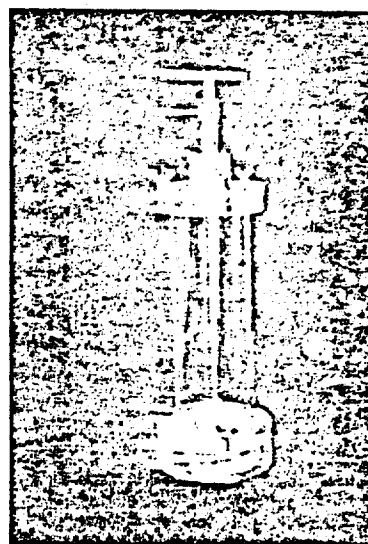


Fig. 2

Plexiglass butter kneader (enlarged 10:1).

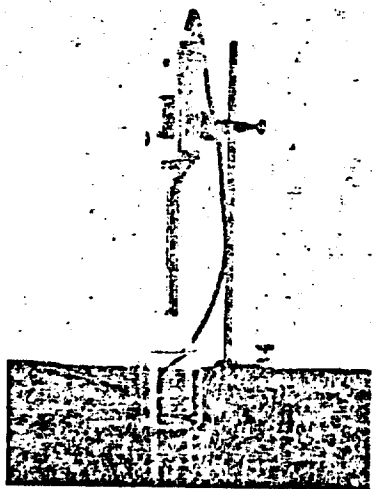


Fig. 3

Ultra Turrax TB 18/2 emulsifier.

In those tests where the behavior of carotene in butter was studied in the presence of definite pro- and antioxidants, the following partially modified method was used.

Emulsification was carried out in an Ultra Turrax TB 18/2 emulsifier operating at 24,000 rpm (Fig. 3). An additional attached device made it possible to perform this process in a nitrogen atmosphere. The ready fat-water emulsion was poured into 4-cm<sup>3</sup> glass tubes, where it immediately solidified without separation into 2 phases.

Butter was subjected to auto-oxidation at 14°C. Part of the samples were exposed to the action of diffuse day light, and another portion was stored in complete darkness.

b) Measurements of Fat Oxidation

The extremely complex process of fat oxidation makes it impossible until now to perform accurate measurements of the rate of progress of these reactions. The only fact known with certainty is the formation of peroxides as the initial sign of a beginning oxidation process, and that aldehydes and ketones are formed during the further course of oxidation. For this reason, chemical methods for determining the oxidation were aimed at the quantitative determination of peroxides, aldehydes and ketones. According to Mohr and Eichst dt (21), none of these methods is superior in its sensitivity to the sensation of our tongue. Similar facts are reported in an abstract by Zeilinger (36), according to which, for example, heptylaldehyde is perceived as irritating already in a concentration of  $1/10^6$  and methylnonyl ketone as rancid in a concentration of  $4/10^6$  by more or less experienced tasters.

A recently developed method for determining peroxides formed, according to Loftus Hills and Thiel (18), Loftus Hills and Wilkinson (19), Stine, Harde-land, Coulter and Jeness (31), and Walser (35), is capable of identifying an

oxidation which has already started before a change in taste has occurred. This method, described below, is based on the oxidation of ferrous chloride to ferric chloride by oxidized fats in a hydrochloric acid benzene-methanol solution, and the formation of a red-colored ferrithiocyanate by reaction with ammonium thiocyanate. The intensity of the red color is measured in a photometer.

The following reagents are required for the peroxide determination by the ferrithiocyanate method:

- 1) A benzene-methanol mixture in a 3:7 ratio.
- 2) A ferrous chloride solution, prepared by dissolving separately, in a 50-cc measuring flask, 0.4g  $\text{BaCl}_2$  and 0.5g  $\text{FeSO}_4$ . After transferring the  $\text{FeSO}_4$  solution into a 100-cc measuring flask, the  $\text{BaCl}_2$  solution is added in a slow rotating motion, followed by 2cc of 10N  $\text{HCl}$ . After the  $\text{BaSO}_4$  precipitate has settled, the clear  $\text{FeCl}_2$  solution is filtered into a small brown flask.
- 3) A standard ferric iron solution. A solution is required, which contains 10g  $\text{Fe}^{+++}$  and 10ml 10N  $\text{HCl}$  in 1000 ml; 48.409g  $\text{FeCl}_3 + 6\text{H}_2\text{O}$  (this amount contains exactly 10g  $\text{Fe}^{+++}$ ) is dissolved in 100ml  $\text{HCl}$  (10N), and this solution is diluted to 1000 ml with distilled water.
- 4) An ammonium thiocyanate solution; 15g ammonium thiocyanate is dissolved in ca. 15 ml distilled water, rinsed into a 50-ml measuring flask and filled up to the mark.

#### Construction of Calibration Lines

Dilution series are prepared from the  $\text{FeCl}_3$  solution in a benzene-methanol mixture, so that 20, 40, 60, 80, 100 and 120 micrograms  $\text{Fe}^{+++}$  are finally present in 20 ml of solution. These dilutions are mixed in a 100-cc Erlenmeyer flask with 0.1cc ammonium thiocyanate reagent, the flask is closed

with a paper-wrapped stopper and rotated (tilted) to achieve a complete mixing, and held exactly 2 min. on a water bath at 50° C for color development. The samples are then cooled 10 min on a water bath at 18° C, and the color intensity then measured photometrically. An Eppendorf photometer was used, and readings were made at a wavelength of 5090 Å, after adjusting the instrument to 100% transmissibility with a pure benzene-methanol mixture. Loftus Hills (25) designates a wavelength of 5050 Å as the absorption peak for ferrithiocyanate, while Walser (43) reports a wavelength of 5150 Å.

In view of the greater possibility of error in the range of a low  $\text{Fe}^{+++}$  concentration, 9 readings each were made at 20 and 40  $\gamma$ /20ml, to readings each at 60  $\gamma$ /20ml, and 3 readings each at 80, 100 and 120  $\gamma$ /20ml.

The average values illustrated in a diagram exhibited a certain amount of scattering and were checked by the compensation and calculation method for conversions with a linear course according to Dannacher (Walser (35)). The formula used for calculating the  $\text{Fe}^{+++}$  amount corresponding to the measured extinction value reads as follows:

$$x' = \frac{y}{0.00503} + 0.00927$$

#### Course of the Investigation

0.25ml of the clear butter oil, which could be obtained by heating and centrifugation at 3000 rpm, was filled up to the marking with the benzene-methanol mixture in a 100-cc glass flask with a ground glass stopper, 0.1ml ammonium thiocyanate and 0.1ml ferrous chloride solution were added, and the mixture was treated further as was already stated during the construction of the calibration curve.



Measurements of a fat-free sample and a reagent-free sample were carried out at the same time, and these two extinction values were subtracted from the first measurement. The resulting difference of the extinctions could be calculated as  $\text{Fe}^{+++}$  with the aid of the above formula.

It is customary to express the peroxide value in milliequivalents of oxygen per kg fat. To calculate this value, the following formula was used:

$$\text{Peroxide value} = \frac{\text{Fe}^{+++}}{\text{g fat} \times 55.84}$$

The individual peroxide values listed in the following table (Table 1) are calculated mean values of 3 measurements.

c) Determination of Carotene

The method of Müller (29) was used, but some modifications were introduced. Since the butter investigated contained only pure  $\beta$ -carotene, a separation (differentiation) into different carotinoids and vitamin A esters on a chromatographic column was not necessary. Carotene losses, which in this method, after chromatography, may amount to 8-12%, were thus avoided in this manner.

The study was carried out as follows: 2-3g butter was dissolved in petroleum ether, the solution was concentrated to a few cubic centimeters by heating under nitrogen, and saponified for 5 min. on a boiling water bath with 10cc 1N ethanolic KOH. The contents were poured into a separating funnel, the glass flask was washed twice with 5cc ethanol each time, and the combined ethanolic extracts were diluted to 50% with distilled water and transferred to a separating funnel. The alcohol-soap mixture was then mixed

with 10cc gasoline (benzine) and shaken strongly for 2 min. After separation of the layers, the ethanolic soap solution was removed, and the carotene-gasoline solution washed with tap water until the wash waters were clear and filtered into a measuring flask over sodium sulfate for drying. The above operations were carried out in a nitrogen atmosphere under exclusion of light. The photometric measurements and evaluation were performed in the same way as described above during the construction of the calibration curve.

#### Personal Studies

In the first test, carotene-free butter was compared with butter containing two different carotene concentrations, which amounted to 8  $\gamma$ /g (corresponding approximately to the maximum carotene content found in natural butter) and 16  $\gamma$ /g. As can be seen from Table 1 and Fig. 4, the peroxide values of carotene-containing butter samples, which were exposed to daylight, were lower, up to the 13th day of storage, than those of the carotene-free comparison butter. However, after 16 days of storage, the peroxide values of the carotene-containing samples were 3 times as high as those of the comparison samples. It is interesting to note that the peroxide values of butter with a carotene content of 16  $\gamma$ /g lay constantly at almost the same distance below the peroxide values of butter with a carotene content of 8  $\gamma$ /g (Fig. 1).

carotene-free

Über die Rolle des  $\beta$ -Carotins bei der Oxydation von Butter. 13

Tabelle 1.

Tabelle 1. Vergleich zwischen carotinfreier Butter und solcher mit einem Carotingehalt von 8  $\gamma$  bzw. 16  $\gamma$ /g

Probenahme am <i>Samplingen</i>	Peroxidwerte <i>Peroxide values</i>		
	carotinfrei	8 $\gamma$ Carotin	16 $\gamma$ Carotin
a) Oxydationsverlauf im diffusen Tageslicht bei 14° C			
<i>day of preparation</i> Tag der Erzeugung	1.152	1.152	1.152
10. Tag	2.146	1.075	0.693
13. " <i>day</i>	2.146	1.533	1.228
16. "	1.842	6.129	5.514
19. "	1.995	6.280	3.373
b) Oxydationsverlauf im Dunkeln bei 14° C			
<i>day of preparation</i> Tag der Erzeugung	1.152	1.152	1.115
10. Tag	1.152	0.920	0.693
13. " <i>day</i>	0.612	0.462	0.231
16. "	1.075	0.639	0.308
19. "	2.146	0.997	0.770

Table 1

Comparison of carotene-free butter with butter containing 8  $\gamma$ /g and 16  $\gamma$ /g carotene.

- a) oxidation in diffuse day light at 14°C.  
b) oxidation in the dark at 14°C.

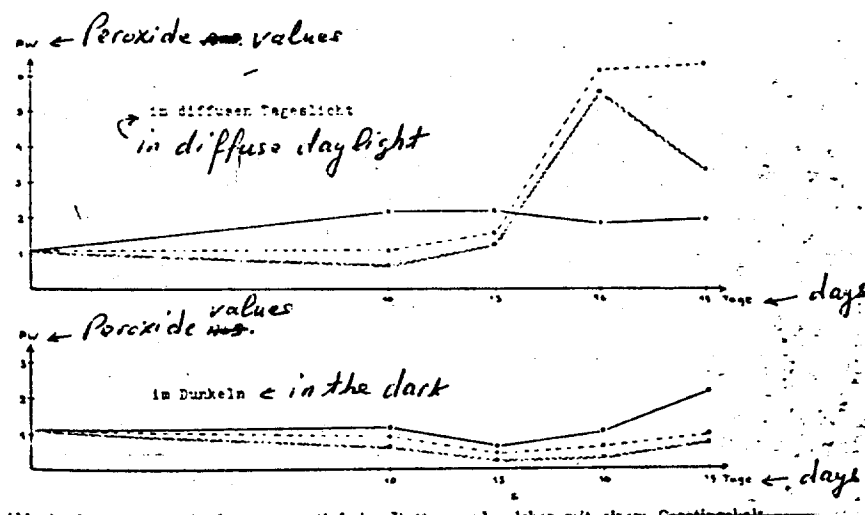


Figure 4

Oxidation course of carotene-free butter and butter with a carotene content of 8 and 16  $\gamma$ /g. — carotene-free; ---- 8  $\gamma$ /g carotene; ~~~~~ 16  $\gamma$ /g carotene

Among the samples stored in the dark, those free of carotene had higher peroxide values in all measurements. In the case of carotene-containing butter, samples with a concentration of 8 $\gamma$ /g showed a greater susceptibility to oxidation than those with a concentration of 16 $\gamma$ /g.

A different course of oxidation was noted after addition of 0.02 mg ascorbic acid to 100g butter, as shown in Table 2 and Fig. 5. These samples and those in the following tests were subjected to oxidation after being enclosed in small glass tubes. The induction period was reduced (shortened) to 6 days, and in the following tests to 3 days. Their peroxide values of carotene-containing samples exposed to light, after a steep increase, were also 40% lower than those of carotene-free comparison samples. During storage in the dark, a slight increase (lengthening) of the induction period was noted. The peroxide values of carotene-containing samples, which on the 9th day of storage were still less than half as great as those of comparison samples, rose rapidly around the 10th day and were 50% higher on the 11th day than those of carotene-free comparison samples. All these observations show a direct participation of carotene during the oxidation of butter.

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Tabelle 2. Oxydationsverlauf nach Zugabe von 0,02 mg Ascorbinsäure/100 g Butter

*Sampling on*

Probenahme am	Peroxidwerte	
	ohne Carotin	mit Carotin

*without carotene*

*Peroxide values*

*with carotene*

*day of preparation*

a) Im diffusen Tageslicht bei 14° C

Tag der Erzeugung	0,539	0,539
6. Tag	1,455	0,770
7. " <i>day</i>	2,376	1,228
9. " <i>day</i>	8,425	4,979

b) Aufbewahrt im Dunkeln bei 14° C

Tag der Erzeugung	0,539	0,539
7. Tag	1,151	0,539
9. " <i>day</i>	1,763	0,616
11. " <i>day</i>	1,455	2,376

Table 2

Oxidation course after addition of 0.02mg ascorbic acid/100g butter.

- a) In diffuse daylight at 14°C.  
b) Stored in the dark at 14°C.

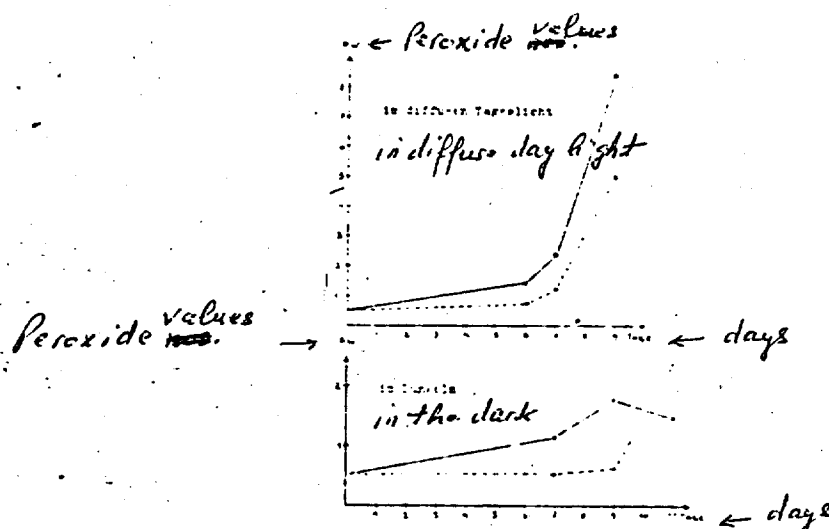
Abb. 5. Oxydationsverlauf nach Zugabe von 0,02 mg Ascorbinsäure/100 g Butter.  
—— carotinfrei, ----- 8  $\gamma$  carotin/g.

Figure 5

Oxidation course after addition of 0.02mg ascorbic acid/100g butter.  
—— carotene-free; ----- 8  $\gamma$  carotene/g.

In order to obtain more precise information on the role of carotene in butter oxidation, both the peroxide values and the corresponding carotene content were determined in the following tests. Copper, diacetyl and lecithin were used as additives, i.e. all products which are known to act as pro-oxidants (oxidation promoters).

### Carotene and Copper

Copper was added in a concentration of 0.03mg/100g as copper lactate. After a short induction period of 3 days (Table 3, Figure 6), the peroxide values of carotene-containing samples rose steeply under the effect of light up to the 5th day, but were still lower, by the almost equal amount of 0.6 milliequivalent oxygen, than the comparison values of carotene-free samples. The carotene content showed, up to the day of a rapid oxidation increase, a relatively steep decline (drop), and from that moment on it dropped only to a slight extent.

16 *without carotene* Wolfgang Schuller *Peroxide values*

Tabelle 3. Oxydationsverlauf nach Zugabe von 0,03 mg Kupfer pro 100 g Butter *with carotene*

<i>Sampling on</i>	Probenahme am	Peroxidwerte		
		ohne Carotin	mit Carotin	% Carotin/g
a) Ausgesetzt dem diffusen Tageslicht bei 14° C				
<i>day of preparation</i> →	Tag der Erzeugung	0,539	0,539	8,04
	3. Tag	2,350	1,753	5,86
	4. " <i>day</i>	4,598	3,982	5,33
	5. "	6,839	6,199	5,23
b) Aufbewahrt im Dunkeln bei 14° C				
	Tag der Erzeugung	0,539	0,539	8,04
	3. Tag	0,612	0,462	6,60
	6. " <i>day</i>	1,760	1,380	6,34
	9. "	2,295	2,145	6,33
	11. "	6,585	4,440	6,23

Table 3

Oxidation course after addition of 0.03mg Cu per 100g butter.

a) Exposed to diffuse daylight at 14°C.

b) Stored in the dark at 14°C.

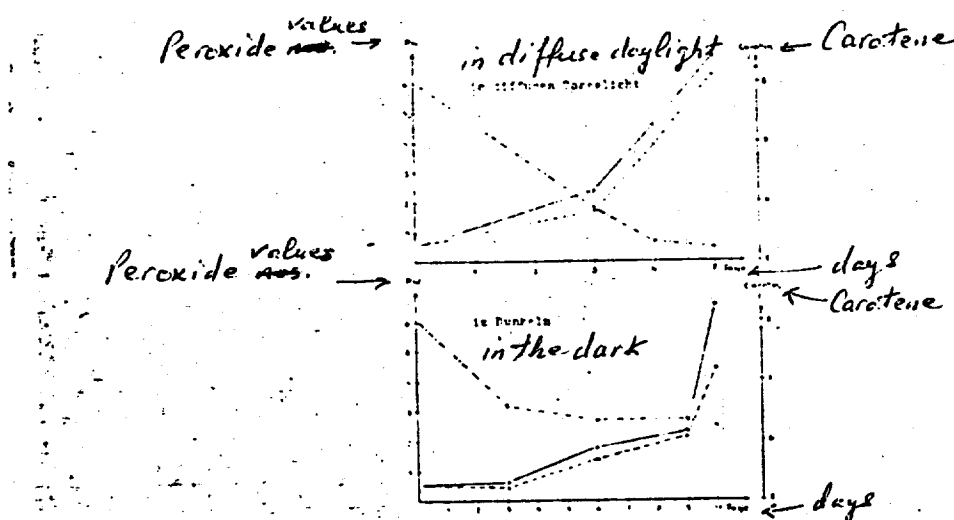


Figure 6

Oxidation course after addition of 0.03mg Cu/100g butter.

—— carotene-free; ----- 8% carotene/g; -.-.-.-. corresponding carotene content.

Samples stored in the dark showed a similar picture, and the induction period was longer. The peroxide values of the carotene-containing samples never exceeded those of carotene-free samples, and, on the contrary, lay at their highest point even below, by 2.1 milliequivalents of oxygen, those of comparison samples. The carotene content again dropped considerably until the day when oxidation greatly increased, but still not in the same measure as in the samples exposed to light. During the steep rise of peroxide values, the losses remained slight.

#### Carotene and Diacetyl

After addition of 0.1 mg diacetyl/100 g butter, the induction period was also equal to 3 days in the samples exposed to light (Table 4, Figure 7), whereby an increased carotene breakdown was noted. From then on, the carotene content remained almost unchanged. The peroxide values of carotene-containing samples were higher by about 0.4 milliequivalent oxygen, than those of the comparison samples only on the 5th day.

*without carotene*  
Wolfgang Schuller  
*Peroxide values*

Tabelle 4. Oxydationsverlauf nach Zugabe von 0.1 mg Diacetyl/100 g Butter

*with carotene*

Samplingen	Probenahme am	Peroxydewerte		
		ohne Carotin	mit Carotin	Carotin/g
a) Ausgesetzt dem diffusen Tageslicht bei 14° C				
<i>day of preparation</i> → Tag der Erzeugung		0,539	0,539	8,04
3. Tag		1,610	0,693	6,53
4. " <i>day</i>		3,142	2,911	6,52
5. "		5,210	5,514	6,50
b) Aufbewahrt im Dunkeln bei 14° C				
Tag der Erzeugung		0,539	0,539	8,04
3. Tag		0,235	0,154	7,68
6. " <i>day</i>		0,539	0,462	7,17
9. "		0,997	1,610	7,23
10. "		0,539	2,068	7,16

*carotene*

Table 4

Oxidation course after addition of 0.1mg diacetyl/100g butter.

a) Exposed to diffuse day light at 14°C.

b) Stored in the dark at 14°C.

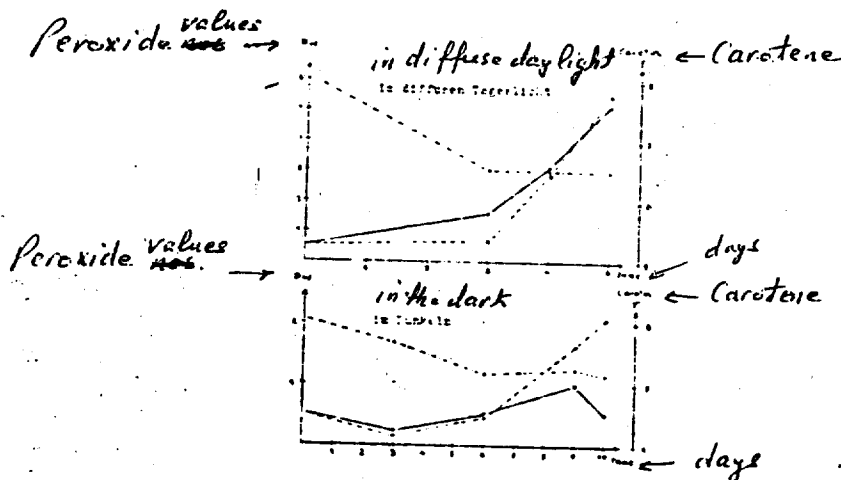


Figure 7

Oxidation course after addition of 0.1mg diacetyl/100g butter.

— carotene-free; ----- 8% carotene/g; ····· corresponding carotene content.



The induction period in the dark lasted for 6 days. Only after this time did the peroxide values of the carotene-containing samples exceed those of comparison samples, and on the 10th day were already 4 times higher. The carotene content dropped in the same manner as in the samples exposed to daylight, only to a lesser extent.

#### Carotene and Lecithin

Lecithin was added in the amount of 0.2g/100g. Under the action of diffuse daylight (Table 5, Figure 8), the peroxide values of carotene-containing samples, after a 3-day long induction period and during the time the oxidation increased, lay below the comparison values by the almost constant amount of 0.7 milliequivalent.

During dark storage, the peroxide values of the carotene-containing samples, on the 3rd day, were lower than those of carotene-free comparison samples by 0.8 milliequivalent, exceeded these values on the 4th day, and on the 5th day of storage were higher by 1 milliequivalent.

In both cases, the carotene breakdown was extensive, but was considerably slower after the peroxide values increased.



### Discussion of Results

A comparison of the curves obtained in the tests discloses some interesting details. By and large (in general), the course of oxidation can be differentiated (separated) into two types according to the mutual position of the curves:

a) The peroxide values of carotene-containing samples are lower during the entire course of oxidation than those of carotene-free comparison samples. The latter thus have a greater susceptibility to oxidation.

Under the effect of light, this fact could be noted in samples containing additions of ascorbic acid, copper and lecithin, while in the dark it was observed only in samples containing copper as an additive.

b) The peroxide values of carotene-containing samples exceed those of carotene-free comparison samples only after a certain period of time has lapsed.

Under the effect of light, such a course was noted in samples containing added diacetyl, while during storage in the dark it was observed in samples containing added ascorbic acid, copper and lecithin.

Reinart (27) used in his tests butter fat enclosed in small glass tubes and subjected this fat at room temperature to the action of diffuse daylight. The amount of carotene added was 0.02%. The Lea method used for peroxide determination is not as sensitive, however, as the ferrithiocyanate method. In spite of this fact, the peroxide values of carotene-containing samples were also lower than those of comparison samples until the 10th day, and exceeded the latter only after the 15th day. A similar agreement with our own studies is noted in the samples containing added diacetyl, although the amount of diacetyl used is considerably greater than the naturally occurring

content of this product in butter. Here also, a lower oxidizability was at first noted in carotene-containing samples, which after 25 days of storage exceeded that of carotene-free comparison samples.

An agreement with the results of our own tests was also noted in diacetyl-containing butter stored in the dark.

Differences are observed merely in time, which can be attributed to the type of treatment of the fat and also the mode of storage. Reinart used pure butter fat without any addition of water, whereas our own studies were performed with butter having an 18% water content.

Thompson and Steenbock (33) used a Warburg respirometer in their studies. In this instrument, a definite amount of fat is subjected to an oxygen pressure at a definite temperature. The manometrically measured pressure drop is taken as a measure of the oxygen absorbed by the fat undergoing oxidation. The experimental amount used in this case was 1g with a carotene addition of 250 , equal to 0.025%, while the maximum content in milk fat is only 0.0008%.

From the increased oxygen consumption of the carotene-containing samples, the above authors concluded that these samples exhibited a higher (greater) oxidizability of fat. However, the results thus obtained indicate merely that fat with a carotene content of 0.025% absorbs more oxygen than carotene-free fat. Still unresolved, however, is the question as to how much of the absorbed oxygen was required for the carotene breakdown, especially since such a breakdown is entirely possible and independent of the fat oxidation at the operating temperature of the Warburg respirometer of 37°C and the oxygen pressure present under these conditions. It is possible that a clearer picture could have been obtained if, along with the amount of oxygen absorbed by the material investigated, the simultaneous carotene breakdown

could also have been determined.

The Warburg respirometer was also used in the tests performed by Schulte and Schillinger (30), the purpose of which was to gain a knowledge of the combined (coupled) autoxidation of fats with vitamin A and its provitamin,  $\beta$ -carotene. The vitamin concentration amounted to  $15 \cdot 10^{-6}$  moles in 2 ml ester. The results of these tests also showed a higher oxygen absorption (uptake) of the carotene-containing samples. The values determined by the above authors could possibly also allow a comparison of the processes taking place during the breakdown of fats on the basis of the extent of vitamin decomposition. Such a determination, however, would be marked by a certain amount of uncertainty, since no exact information is yet available on the stoichiometric ratio of the reaction between vitamin A and oxygen, and also because we have to assume that certain secondary reactions playing apart in oxygen consumption are taking place.

The following tabulated data provide an interesting insight into this problem.

Oxygen uptake and vitamin A acetate decrease (drop) of a solution of vitamin A acetate in a 1:1 mixture of laurate and oleate esters at different vitamin A concentrations after 6 hours. (Copy headings and figures in table at bottom of p. 21).

The percentage vitamin losses are the same in all 3 cases. However, when converted to individual vitamin concentrations, these amounts would be equal to  $2.1 \cdot 10^{-6}$ ,  $4.5 \cdot 10^{-6}$  and  $8.7 \cdot 10^{-6}$  moles/batch. This progressive vitamin A decrease is contrasted, however, only with a linearly higher oxygen uptake. It could be assumed that the resulting oxygen requirement for vitamin A oxidation is not fully satisfied (met) by the further uptake of about 20 microliters, and for this reason less oxygen is available for fat oxidation.

Similar observations were made during our own tests (see Fig. 4), where the peroxide values of samples with a carotene concentration of 16  $\gamma$ /g butter were always lower than the peroxide values of samples with a carotene content of 8  $\gamma$ /g butter.

The carotene determinations performed in our own studies, along with the measurement of peroxide values, provided a possibility of comparison with the already mentioned results obtained by Holmann (10).

The investigations carried out in the present study were concerned only with a determination of the first signs of oxidation, namely peroxides. However, also during this stage, considerable carotene losses could already be noted. These losses amounted to 20-35% in samples exposed to the action of daylight, and to 11-23% in samples stored in the dark. In all cases, however, the peroxide values increased only after a relatively great drop of the carotene content had taken place.

Diemayr et al. (5) used model esters in their determinations; 5 cm<sup>3</sup> of the ester, with 2.5 mg  $\beta$ -carotene, was aerated with oxygen at 94° C. In view of the high temperature and the strong effect of oxygen, it is entirely possible that, in regard to peroxide formation, a similar situation could have occurred after 30 and 60 minutes as the one encountered in our own samples stored in daylight (Fig. 4) only on the 16th day of storage. Determination of the oxidizability was done by determining the peroxide numbers according to Lea. According to Walser (35), however, a certain amount of subjectivity in this method cannot be denied, and the method also does not possess the required sensitivity, as was already stated above.

According to the results obtained by Hinko (9), with the additional uptake of oxygen in fat in the presence of carotene, a breakdown of the latter takes place simultaneously. The above author further reports practical experience showing that carotene provides a certain amount of protection against oxidation

in stored fats.

An interesting possibility of comparison is provided by the study of Chevalier (4), who, as was reported above, found that carotene exerts a pro-oxidant effect under the action of light and an antioxidant effect in the dark. These results were obtained by subjecting 10 mg triolein to oxidation in a thin layer at 40° C. Some of the samples contained 50-80  $\gamma$  of  $\beta$ -carotene. The effect of light was achieved by using a mercury vapor lamp, restricted with a filter to a wavelength of 3400 Å. The samples stored in the dark gave the following results:

<u>Hours</u>	1	2	3	4	5	10	15	17
<u>Triolein &amp; carotene</u>	0.87	0.79	0.76	0.71	0.68	0.71	0.74	0.80
<u>Triolein</u>								

The oxidation quotient of samples stored in the dark shows the antioxidant effect of carotene, which decreases, however, with increasing duration of time (in the same way as in Fig. 4). On the other hand, the oxidation quotients of samples stored under light exposure were:

<u>Hours</u>	1	2	3	4	5	6	7	8
<u>Triolein &amp; carotene</u>	1	1.17	1.25	1.28	1.30	1.29	1.19	1.26
<u>Triolein</u>								

We must assume that, as a result of the very intensive irradiation, the oxidation values of carotene-containing samples reach already after 1 hour, the same level as that of carotene-free comparison samples, and then start to rise later, a condition which occurs only on the 14th of storage with samples of our own test (Fig. 4), which were stored at a much lower temperature under the effect of diffuse day light.

Summary

1. In order to determine whether or to what extent  $\beta$ -carotene plays a role in the oxidation of butter, comparative studies were carried out on model butter, with or without carotene and with or without different additives.

2. In butter without any kind of oxidation-promoting additive, an oxidation inhibiting effect of carotene was noted until the 13th day during storage at about 14° C in diffuse daylight. Only from the 13th day on, did carotene exert a pro-oxidant effect. In the dark, the antioxidant effect of carotene could be detected during the entire investigation period of 19 days.

When samples with different carotene concentrations were used, those with a lower carotene content had a higher susceptibility to oxidation.

3. During the induction period, in all samples tested, the peroxide values of carotene-containing samples were lower than those of carotene-free comparison samples.

4. Samples containing added ascorbic acid, lecithin or copper consistently exhibited an oxidation-inhibiting effect in daylight. In the dark, such an effect of carotene was noted only in samples containing added copper.

5. An initially oxidation-inhibiting, but later an oxidation promoting, action of carotene was noted in butter samples containing added diacetyl, both during storage in daylight and in the dark; a similar behavior in samples containing added ascorbic acid or lecithin was noted only in the dark.

6. Carotene breakdown was intense during the induction period, but diminished during the course of progressive fat oxidation.

7. From the above facts, we might draw the conclusion that carotene represents a protective factor for fat during the induction period by acting in such a way that it protects fat from oxidation by capturing oxygen activated by light energy or various oxidation promoting substances, but at the same time



itself undergoes a partial breakdown. It is possible that only the breakdown products thus formed become involved as oxidation factors in the further processes of fat deterioration.

Bibliography

(See pages 24-26)

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## Über die Rolle des $\beta$ -Carotins bei der Oxydation von Butter

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### Einleitung

Verschiedene Substanzen, die durch die Art der Milchbehandlung Butterbestandteile geworden sind, wie auch solche, die in der Milch und im MilCHFett natürlich vorkommen, beeinflussen den Ablauf der Autoxydation des Butterfettes. Je nach der Art ihrer Wirkung spricht man von Pro- oder Antioxydantien. Zahlreiche wissenschaftliche Arbeiten beschäftigen sich mit dem oxydationshemmenden und -fördernden Verhalten dieser Stoffe. Die Forschungsergebnisse über die oxydative Mitwirkung verschiedener Vitamine widersprechen jedoch einander.

Aufgabe dieser Arbeit sollte es sein, die Frage, in welcher Weise das  $\beta$ -Carotin an der Butterfett-Autoxydation Anteil hat, einer Klärung näherzubringen.

### Stand der Frage und Problemstellung

Das Wesen der Butteroxydation besteht in der Anlagerung von Sauerstoff an die Doppelbindungen der ungesättigten Fettsäurereste durch Bildung von Peroxyden und in der darauffolgenden Spaltung der Fettsäureketten unter Bildung von Aldehyden und Ketonen. Der Gehalt an Fettsäuren mit doppelt gebundenen C-Atomen gilt als primärer Faktor der Oxydierbarkeit. Diese Fettsäuren zeigen infolge ihrer Ungesättigtheit eine große Reaktionsbereitschaft. Sie werden also in

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erster Linie oxydiert und oxydieren in einer Art Kettenreaktion die anderen Fettsäuren mit auf.

Diese katalytischen Vorgänge werden nach Greenbank (7) durch die Anwesenheit freier Fettsäuren gefördert. Von den gesättigten Fettsäuren gelten hochmolekulare als gefährlicher als niedermolekulare.

Thome (32) und Mattson (20) fanden Zusammenhänge zwischen dem Gehalt der Butter an ungesättigten Fettsäuren und der Länge der Oxydations-Induktions-Periode. Zwischen dem Grad der Oxydierbarkeit und dem prozentuellen Gehalt an ungesättigten Fettsäuren konnten keine direkten Zusammenhänge festgestellt werden.

Schmalfuß (29) machte die Feststellung, daß auch Fette, die ausschließlich aus gesättigten Fettsäureanteilen bestehen, der Oxydation anheimfallen können. Die Induktionsperiode war von entsprechend längerer Dauer.

Reinart (26) macht für die Oxydationsanfälligkeit des Butterfettes die hochungesättigten Fettsäuren der Reihe  $C_{20}-C_{22}$  verantwortlich.

Der Oxydation des Butterfettes durch Licht liegt eine Art photochemischer Vorgänge zugrunde, die bei quantenhafter Absorption des Lichtes folgende von Böhmer und Großfeld (1) veranschaulichte Kettenreaktion einleitet.

1.  $F + h\nu \rightarrow F^*$
2.  $F^* + O_2 \rightarrow F'O_2$
3.  $F'O_2 + F \rightarrow FO_2 + F'$

Das Fettsäuremolekül (F) wird durch eingestrahle Lichtenergie ( $h\nu$ ) zur Sauerstoffanlagerung ( $F'O_2$ ) aktiviert. Dieses angeregte Peroxyd gibt die Reaktionsenergie weiter und wird so zum normalen Peroxyd.

Nur ungesättigte und aromatische Verbindungen können nach Eggert (6) im sichtbaren und ultravioletten Wellenbereich das Licht absorbieren. Anregbare Elektronen befinden sich an den chromophoren

Gruppen, deren eine Gattung, die Äthylengruppe  $-C=C-$ , Baustein der ungesättigten Fettsäuren, aber auch des Carotins ist.

Nach Schmalfuß (29) erreichen ungesättigte Fettsäuren bei Bestrahlung mit Licht von einer Wellenlänge unterhalb 4100 Å eine deutliche Oxydationsanfälligkeit.

Der Oxydationsbeschleunigung durch Lichtwirkung unterliegen besonders konjugierte Fettsäuren. Lembke (16) gelang es, konjugierte Fettsäuren in der Butter nachzuweisen und zu bestimmen.

Pont (23, 25) stellte in von direktem Sonnenlicht und diffusem Tageslicht bestrahlten Milchproben die oxydige Geschmacksverschlechterung fest.

Der Sauerstoffgehalt der Butter ist ebenfalls von großer Bedeutung für ihre Oxydationsanfälligkeit. Entgegen der früheren Ansicht, daß das dispergierte Butterwasser in erster Linie als Sauerstoffträger in Frage

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käme, zeigten Untersuchungen von Loftus Hills (17), daß Sauerstoff in flüssigem Butterfett zu 2,55 ml/100 g Fett löslich ist. Plastische Butter enthält bis zu 55% flüssiges Fett.

Chalmers (3) weist besonders auf die verschiedenen Milchbehandlungsarten (Pasteurisieren, Evaporieren) hin, die den Sauerstoffgehalt des Milchfettes und damit der Butter verändern können. Er verweist ferner auf die große Bedeutung der reduzierenden Bakterien, durch deren biologischen Sauerstoffbedarf nur mehr wenig  $O_2$  für den chemischen Sauerstoffbedarf zur Verfügung steht.

Das Butterwasser kann in seiner Funktion als Sauerstoffträger, ferner aber wegen der Möglichkeit des Gehaltes an Schwermetallspuren und verschiedenen prooxydant wirkenden Salzen in den Oxydationsablauf wirksam eingreifen.

Greenbank (7) zeigte, daß Butter mit niedrigem Flüssigkeitsgehalt zur Aldehydbildung und eine solche mit einem höheren Gehalt zur Säurebildung neigt.

Die prooxydante Wirkung von Natrium- und Magnesiumchlorid konnte Loftus Hills (17) nachweisen.

Infolge des Kontaktes von Milch und Milchfett kann der Gehalt an Metallionen über das normale Maß hinaus anwachsen.

Besondere Bedeutung kommt den Schwermetallspuren zu. Von allen Metallen gilt Kupfer als der stärkste Sauerstoffüberträger. Nach Chalmers (3) wirken die Kupfer-Ionen, die sich um das Fettkügelchen lagern oder in der Butter dispers verteilt sind, den Sauerstoff aktivierend, als Katalysator.

Beobachtungen ähnlicher Art machten Tollenaar (34), Greenbank (7), Pont (24) und Krukowsky (14).

Natürliche Bestandteile können als Oxydationsregler oder Katalysatoren maßgebend am Oxydationsablauf beteiligt sein. Von diesen seien zunächst die Phospholipide erwähnt. Ihr Gehalt in der Butter liegt bei 0,02%. Sie umgeben das Fettkügelchen nach King (11) in einer geschlossenen Schicht so, daß ihre Fettsäureanteile in die Fettphase reichen und die hydrophilen OH-Gruppen der Phosphorsäure gegen die umgebende wäßrige Phase gerichtet sind. Aus dieser Anordnung kann der Schluß gezogen werden, daß die Phospholipide als Sauerstoffüberträger aus der wäßrigen Phase in die Fettphase fungieren.

Auch fallen sie nach Greenbank (7) wegen ihres Gehaltes an hoch-ungesättigten Fettsäuren leicht der Oxydation anheim.

Lea (15) mißt den Phospholipiden als Prooxydantien des Milchfettes ebenfalls Bedeutung zu. Allerdings seien sie nur als sogenannter Lipoproteinkomplex befähigt, wesentlich in die Oxydationsvorgänge einzugreifen.

Im dispergierten Zustand sind sie unwirksam. In der Butter treten sie wegen ihres geringen Vorkommens gegenüber hochungesättigten

Fettsäuren kaum in Wirkung. Es konnte sogar in Verbindung mit Tocopherol eine oxydationshemmende Wirkung festgestellt werden.

Tocopherol selbst gilt als Antioxydant. Seine oxydationshemmende Wirkung zeigten unter anderen Schulte und Schillinger (30) in ihren Versuchen über die Oxydation von Vitamin A und Carotin.

Nach Reinart (28) besitzt Tocopherol eine oxydationshemmende Wirkung. Diese tritt aber wegen der niedrigen Konzentration in der Butter (2,5 g/100 g) kaum merklich in Erscheinung.

Krukowsky (13) und Mitarbeiter fanden eine Oxydationsresistenz bei Milchfett durch 2,614 g Tocopherol/100 g Fett trotz Zugabe von 1 mg Kupfer/l.

Bei der Säuerung des Butterungsgutes wird von gewissen aromabildenden Bakterien das Diacetyl gebildet. Es ist ein Diketon, das sehr leicht reduziert wird und aus diesem Grunde seine Umgebung oxydiert.

Neben einem oxydativen Angriff auf die Butter stellte Reinart (27) auch einen solchen auf Butterfarbstoffe, wie Carotin und Bixin, fest.

Nach King (12) reagiert Diacetyl als Wasserstoffakzeptor. Wasser fungiert als Wasserstoffdonator, während ungesättigte Fettsäuren und Butterfarbstoffe den Sauerstoff aufnehmen.

Gyllenberg (8) untersuchte die Wirkung des Vitamin C in Milchprodukten. Den Ergebnissen zufolge liegt wahrscheinlich der Grund für die Bereitschaft des Vitamin C, die Oxydation zu hemmen, in seiner leichten Oxydierbarkeit zur Dehydroascorbinsäure.

Ergebnisse von Greenbank (7) stimmen damit überein. Ascorbinsäure bietet besonders gegen durch Metallinfektion hervorgerufenen Oxydation Schutz.

Krukowsky (14) und Mitarbeiter lieferten wertvolle Einblicke in die Art des Ablaufes der Oxydation von Ascorbinsäure zur Dehydroascorbinsäure und deren Zusammenspiel mit der Oxydation des Fettes. Nur ein bestimmtes Mengenverhältnis zwischen Ascorbinsäure und gelöstem Sauerstoff vermochte das Auftreten des Oxydationsgeschmackes zu verhindern.

Großes Interesse erweckt das oxydative Verhalten des Vitamin A. Besonderes Augenmerk galt aber der Wirkung des  $\beta$ -Carotins.

Thompson und Steenbock (33) führten Versuche über die gekuppelte Carotin- und Fettoxydation in Schmalz, Olivenöl, Baumwollöl und Sojaöl durch. Gefunden wurde eine Verkürzung der Induktionsperiode der carotinhaltigen Proben gegenüber jener der Vergleichsproben um 20 bis 40 Stunden.

Reinart (26) stellte bis zum 10. Tag der Versuchsdauer eine antioxydante und von diesem Zeitpunkt an eine prooxydante Wirkung des Carotins fest.

Schulte und Schillinger (30), die das Verhalten des Vitamin-A-acetats und des Carotins bei der Fettoxydation prüften, fanden einen

erhöhten Sauerstoffverbrauch des Fettes in Gegenwart von Vitamin A und Carotin.

Holmann (10) verfolgte den Ablauf der Oxydation von Linolsäuremethylester und den gleichzeitigen Carotinabbau photometrisch. Er konnte feststellen, daß nahezu das gesamte Carotin abgebaut worden ist, ehe 10% dieses Esters oxydiert sind.

Diemayr und Mitarbeiter (5) führten ihre diesbezüglichen Untersuchungen ebenfalls an Modellestern durch. Gefunden wurde ein Ansteigen der Peroxydzahlen der carotinhaltigen Proben gegenüber denen der carotinfreien Kontrollproben um 400%.

Hinko (9) verfolgte neben anderen Farbstoffen auch die oxydative Einwirkung des Carotins und stellte bei dessen Anwesenheit eine erhöhte Sauerstoffaufnahme fest.

Chevalier und Mitarbeiter (4) fanden ein antioxydantes Verhalten des Carotins in der Dunkelheit und ein prooxydantes Verhalten bei Lichteinwirkung.

Chalmers (3) zufolge kommt dem Carotin beim Fettverderb eine oxydationshemmende Wirkung zu.

Die teils sich widersprechenden Ansichten, gewonnen auf Grund verschiedener und vielleicht auch nicht immer glücklich gewählter Methoden und Verfahren, gaben Anlaß, die folgenden Untersuchungen in Angriff zu nehmen.

Die Versuche sollten an reinem Butterfett vorgenommen werden, und die zugesetzten Carotinnengen nicht die natürliche, in der Butter vorkommende Konzentration übersteigen. Letztlich sollte eine empfindliche Methode der Peroxydmessung diese Frage klären helfen.

### Methodischer Teil

#### a) Herstellung einer carotinfreien Butter

Zur Ermöglichung eines genauen Studiums über die Rolle des Carotins bei der Oxydation der Butter erscheint es zunächst notwendig, eine Butter, frei von Carotin, Diacetyl und Phospholipiden, zu erzeugen. Dieses Produkt sollte aber auch, insbesondere in seinen sonstigen chemischen und physikalischen Eigenschaften, wie z. B.: gleiches Mengenverhältnis der Fettsäureanteile, Wassergehalt, Wasserverteilung und Struktur, einer normalen Molkereibutter gleichen.

Es bestand also nur die Möglichkeit, frische Butter mit möglichst schonenden, oxydationsneutralen Mitteln so zu behandeln,

daß sie den verlangten Erfordernissen entsprach. Die rein chemische Entfernung des Carotins gelingt nur durch Verseifung des Fettes. Eine Rückführung der Seifen in Butterfett wäre aus Gründen der Fettschonung nicht mehr angebracht gewesen. Jedoch gelang es, nach Böhmer und Großfeld (2), das Carotin durch Adsorption an oberflächenaktive Stoffe zu binden. Diese Methode

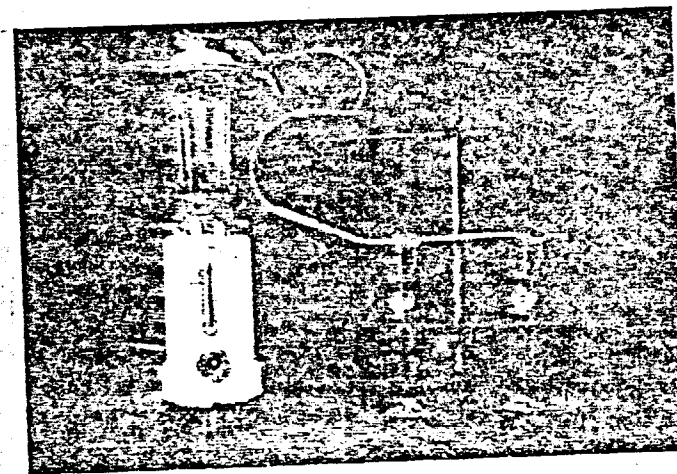


Abb. 1. a Waschflasche mit Pyrogallol und Kalilauge zur Reinigung des Stickstoffes.  
b Apparat zur Herstellung der Emulsion

wird häufig zur Klärung von Fett verwendet. Hand in Hand geht damit eine Entfernung der Phospholipide und des Tocopherols. Diese Methode erscheint im Prinzip als für den vorliegenden Zweck geeignet.

Die Ausgangsbutter wurde aus frischem Süßrahm von 30% a Fett in einem Alfa-Sturzbutterraß aus Glas erzeugt. Die Bauweise des Fasses verhindert den Kontakt des Butterungsgutes mit Metallteilen. Um die Evakuierung des Butterfasses und das Einblasen von Stickstoff nach der Fällung zu ermöglichen, wurde eine Zusatzvorrichtung angebracht. Ferner wurden zum Schutz vor Lichteinwirkung die Glasteile schwarz verkleidet.

Gebuttert wurde bei einer Temperatur von  $14^{\circ}\text{C}$ , bis das Korn Erbsengröße aufwies. Nach Ablassen der Buttermilch wurde das Butterkorn mit frischem Leitungswasser bis zur völligen Klarheit des Waschwassers gewaschen, unmittelbar darauf in einem verschließbaren Glasgefäß mit 5% pulverisierter Aktivkohle

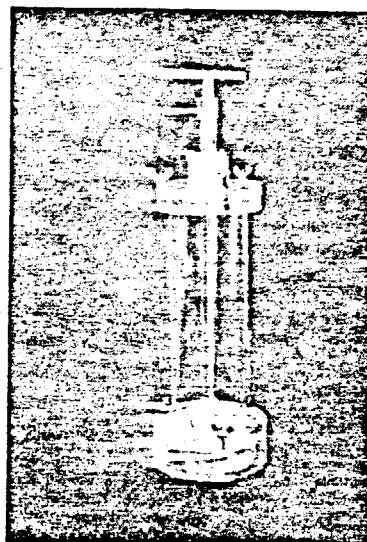


Abb. 2. Butterknetter aus Plexiglas (Verkleinerung 10:1)

(„medicinalis“) vermengt und im Wasserbad auf  $95^{\circ}\text{C}$  erhitzt. Bei dieser Temperatur wurde das Butter-Aktivkohle-Gemisch 20 Min. unter häufigem Rühren und Schütteln gehalten. Da sich die Trennung des Butter-Aktivkohle-Gemisches durch Filtration angesichts des relativ hohen Gehaltes an Aktivkohle bald als fast unmöglich herausstellte und zudem hohe Fettverluste mit sich brachte, wurde versucht, eine grobe Vortrennung von Butterfett und Aktivkohle in der Zentrifuge zu erreichen. Zur Verfügung stand eine Gerber-Milchzentrifuge mit ca. 1000 Umdrehungen/Min. Als Zentrifugenröhrchen wurden Reagenzgläser von  $100\text{ cm}^3$  Inhalt verwendet und

ein hierzu passender Einsatz angefertigt. Die Einhaltung der als zweckmäßig befundenen Arbeitstemperatur von  $35^{\circ}\text{C}$  gewährleistete eine aufgesetzte elektrische Heizspirale. Auf diese Weise gelang es, bei einer Zentrifugierungsdauer von 20 Min. eine Grobtrennung von Aktivkohle und Butterfett herbeizuführen. Die anschließende

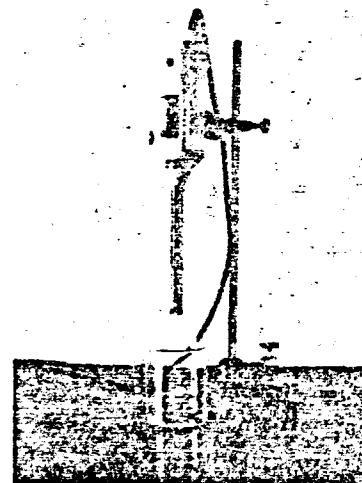


Abb. 3. Ultra Turrax TB 18.2 Emulgator

Feintrennung erfolgte in einem Doppelmantel-Heißwassertrichter mittels eines Faltenfilters Schleicher & Schüll Nr. 588.

Carotin ( $\beta$ -Carotin crist., synth. Hoffmann-La Roche) wurde aus frisch erbrochener Phiole dem Fett direkt zugesetzt.

Die genau gewogene Fettmenge wurde in einem Bauknecht-Mixapparat (Abbildung 1), ausgestattet mit einer Vorrichtung zum Evakuieren und Einblasen von Stickstoff, mit der berechneten Menge an destilliertem Wasser emulgiert. Dieser Vorgang erfolgte im dauernden Stickstoffstrom bei einer Temperatur von  $40^{\circ}\text{C}$ . Um die Trennung der Emulsion in eine Fett- und Wasserphase während

des Auskühlens zu verhindern, wurde ein besonderer Knetapparat verwendet (Abbildung 2), der den Knetvorgang unter Stickstoffatmosphäre ermöglichte. Dieser Knetapparat war aus Plexiglas derart gefertigt, daß kein Metallteil mit der Butter in Berührung kam. In diesem Apparat wurde die Fett-Wasser-Emulsion unter dauerndem Kneten in Stickstoffatmosphäre zum Erstarren gebracht, hierauf ausgestoßen, mit einem Plexiglasspatel portioniert, in Pergament verpackt und so der Autoxydation ausgesetzt.

Bei denjenigen Versuchen, bei denen das Verhalten des Carotins in Butter bei Anwesenheit bestimmter pro- und antioxydant wirkender Stoffe studiert wurde, gelangte eine teilweise abgeänderte Methode zur Anwendung:

Der Emulgierungsvorgang erfolgte mittels eines Ultra Turrax TB 182 Emulgators mit 24.000 Umdrehungen/Min. (Abbildung 3). Eine zusätzliche Apparatur ermöglichte diesen Vorgang in Stickstoffatmosphäre. Die fertige Fett-Wasser-Emulsion wurde in Glasröhrchen mit einem Inhalt von 4 cm<sup>3</sup> gegossen. Sie erstarrte unmittelbar darauf, ohne sich in die zwei Phasen zu trennen.

Die Butter wurde bei 14°C der Autoxydation ausgesetzt. Ein Teil der Proben stand unter der Einwirkung von diffusem Tageslicht, ein anderer war in völliger Dunkelheit aufbewahrt.

#### b) Messung der Fettoxydation

Der äußerst komplizierte Vorgang der Fettoxydation macht bis heute eine exakte Messung der Fortgeschrittenheit dieser Reaktionen unmöglich. Man weiß nur mit Sicherheit, daß das Anfangszeichen eines beginnenden Oxydationsvorganges die Bildung von Peroxyden ist, und daß im weiteren Verlaufe Aldehyde und Ketone auftreten. Die chemischen Oxydationsbestimmungsmethoden waren daher auf die quantitative Ermittlung der Peroxyde bzw. der Aldehyde und Ketone gerichtet. Nach Mohr und Eichstädt (21) sollte keine dieser Methoden an Empfindlichkeit die Sinnesindrücke unserer Zunge übertreffen. Ähnlich referiert Zeilinger (36), wonach beispielsweise von einigermaßen geübten Kostern Heptylaldehyd bereits in einer Konzentration von 1 zu 1 Million als kratzend, Methylonylketon in einer Konzentration von 4 zu 1 Million als ranzig empfunden wird.

Eine neuerdings entwickelte Methode zur Feststellung der gebildeten Peroxyde nach Loftus Hills und Thiel (18), Loftus Hills und Wilkinson (19), Stine, Hardeland, Coulter und Jeness (31) und Walser (35) weist eine bereits eingetretene Oxydation nach, ehe noch eine Geschmacksänderung aufgetreten ist. Diese unten beschriebene Methode beruht darauf, daß durch aufoxydierte Fette in einer salzsauren Benzol-Methanol-Lösung Ferrochlorid zu Ferrichlorid oxydiert wird und mit bereits vorgelegtem Ammoniumthiocyanat das rotgefärbte Ferrithiocyanat ergibt. Die Intensität der Rotfärbung wird photometrisch gemessen.

Als Reagenzien zur Peroxydbestimmung nach der Ferrithiocyanatmethode sind erforderlich:

1. Benzol-Methanol-Gemisch im Verhältnis 3 : 7.

2. Ferrochloridlösung.

0,4 g Bariumchlorid und 0,5 g Ferrosulfat werden je für sich in einem 50-cm<sup>3</sup>-Meßkölbchen gelöst. Nach Überführung der Ferrosulfatlösung in einen 100-cm<sup>3</sup>-Meßkolben wird die Bariumchloridlösung unter langsam rotierender Bewegung zugefügt und noch anschließend 2 cm<sup>3</sup> 10 n Salzsäure. Nach Absetzen des Bariumsulfatniederschlags wird die klare Ferrochloridlösung in ein braunes Fläschchen gefiltert.

3. Standard-Ferrieisenlösung.

Verlangt wird eine Lösung, die 10 g Fe<sup>+++</sup> und 100 ml 10 n HCl in 1000 ml enthält. 48,409 g FeCl<sub>3</sub> · 6 H<sub>2</sub>O (diese Menge enthält genau 10 g Fe<sup>+++</sup>) werden in 100 ml HCl (10 n) aufgelöst und diese Lösung mit destilliertem Wasser auf 1000 ml aufgefüllt.

4. Ammoniumthiocyanatlösung.

15 g Ammoniumthiocyanat werden in zirka 15 ml destilliertem Wasser aufgelöst, in einen 50-ml-Meßkolben gespült und bis zur Marke aufgefüllt.

#### Aufstellung der Eichgeraden

Von der FeCl<sub>3</sub>-Lösung werden in Benzol-Methanol-Gemisch Verdünnungsreihen angelegt, so daß zuletzt in 20 ml der Lösung 20, 40, 60, 80, 100 und 120  $\gamma$  Fe<sup>+++</sup> enthalten sind. Diese Verdünnungen werden in 100-cm<sup>3</sup>-Erlenmeyerkolben mit 0,1 cm<sup>3</sup> Ammoniumthiocyanatreagens versetzt, der Kolben mit einem papierumwickelten Stopfen verschlossen, zur vollständigen Mischung geschwenkt und genau 2 Min. im Wasserbad bei 50°C zur Farbentwicklung behalten. Hierauf kühlen die Proben in einem Wasserbad von 18°C 10 Min. lang aus und werden anschließend photometriert.

Für die Photometrierung stand ein Photometer Eppendorf zur Verfügung. Die Ablesung erfolgte bei einer Wellenlänge von 5090 Å, nachdem das Gerät bei reinem Benzol-Methanolgemisch auf 100% Durchlässigkeit adjustiert worden war. (Lottus Hills (25) bezeichnet als Absorptionsmaximum bei Ferrithiocyanat eine Wellenlänge von 5050 Å, Walser (43) hingegen eine solche von 5150 Å.)

Wegen der größeren Fehlermöglichkeit im Bereiche einer niedrigen  $\text{Fe}^{+++}$ -Konzentration wurden bei

20 und 40  $\gamma$ /20 ml je 9 Ablesungen,

60  $\gamma$ /20 ml je 6 Ablesungen,

80, 100 und 120  $\gamma$ /20 ml je 3 Ablesungen

durchgeführt.

Die bei der graphischen Aufzeichnung veranschaulichten Mittelwerte zeigten eine gewisse Streuung und wurden nach dem Ausgleichs- und Berechnungsverfahren für linear verlaufende Umsetzungen nach Dannacher (Walser (35)) geprüft. Die Formel zur Berechnung einer der gemessenen Extinktion entsprechenden  $\text{Fe}^{+++}$ -Menge lautet demnach:

$$x = \frac{y}{0,00503} \div 0,00927.$$

#### Untersuchungsgang

0,25 ml des klaren Butteröles, welches durch Erhitzen und Zentrifugieren bei 3000 Umdrehungen/Min. erreicht werden konnte, wurden mit dem Benzol-Methanol-Gemisch in einem 100-cm<sup>3</sup>-Glaskolben mit eingeschlifffenem Glasstopfen bis zur Marke aufgefüllt, 0,1 ml Ammoniumthiocyanat- und 0,1 ml Ferrochloridlösung hinzugefügt und weiter behandelt, wie bereits bei der Aufstellung der Eichkurve erwähnt worden ist.

Die Messung einer fettlosen Probe und einer reagenzlosen Probe wurde mit durchgeführt und diese beiden Extinktionen von der ersteren in Abzug gebracht. Die daraus resultierende Differenz der Extinktionen konnte mit Hilfe der vorher aufgestellten Formel in  $\text{Fe}^{+++}$  umgerechnet werden.

Es ist gebräuchlich, den Peroxydwert in Milliäquivalent Sauerstoff pro kg Fett auszudrücken. Zu dessen Berechnung diente folgende Formel:

$$\text{Peroxydwert} = \frac{\text{Fe}^{+++}}{\text{g Fett} \times 55,84}.$$

Die in der folgenden Tabelle aufscheinenden einzelnen Peroxydwerte sind errechnete Mittelwerte aus je drei Messungen.

#### c) Bestimmung des Carotins

Verwendet wurde die Methode nach Müller (29). Es wurden jedoch einige Modifizierungen vorgenommen. Da die zu untersuchende Butter nur reines  $\beta$ -Carotin enthielt, war eine Differenzierung in der chromatographischen Säule in verschiedene Carotinoide und Vitamin-A-Ester nicht notwendig. Carotinverluste, welche bei diesem Bestimmungsverfahren nach der Chromatographierung 8 bis 12% betragen können, wurden auf diese Weise hintangehalten.

Der Gang der Untersuchung war demnach folgender:

2 bis 3 g Butter wurden in Petroläther gelöst, die Lösung unter Stickstoffatmosphäre durch Erwärmung auf wenige cm<sup>3</sup> eingeeengt, mit 10 cm<sup>3</sup> in äthanolischer Kalilauge auf dem siedenden Wasserbad 5 Min. lang verseift, der Inhalt in einen Scheidetrichter gegossen, der Glaskolben zweimal mit je 5 cm<sup>3</sup> Äthanol gewaschen, die vereinigten äthanolischen Extrakte mit destilliertem Wasser auf 50% verdünnt und in den Scheidetrichter übergeführt. Hierauf wurde das Alkohol-Seifen-Gemisch mit 10 cm<sup>3</sup> Benzin versetzt und 2 Min. heftig geschüttelt. Nach der Trennung der Schichten wurde die äthanolische Seifenlösung abgelassen, die Carotin-Benzin-Lösung bis zur Klarheit des Waschwassers mit Leitungswasser gewaschen und über Natriumsulfat zur Trocknung in eine Mensur gefiltert. Diese Vorgänge geschahen unter Lichtabschluß in Stickstoffatmosphäre. Die photometrische Messung und Auswertung erfolgte, wie bereits bei der Aufstellung der Eichkurve beschrieben worden ist.

#### Eigene Untersuchungen

Beim ersten Versuch wurde carotinfreie Butter solcher mit zwei verschiedenen Carotinkonzentrationen gegenübergestellt. Diese

Über die Rolle des  $\beta$ -Carotins bei der Oxydation von Butter 13  
**Table 1.**  
 Tabelle 1. Vergleich zwischen carotinfreier Butter und solcher mit einem Carotingehalt von 8  $\gamma$  bzw. 16  $\gamma$ /g

Probenahme am <i>Sampling on</i>	Peroxydwerte <i>Peroxide</i>		
	carotinfrei	8 $\gamma$ Carotin	16 $\gamma$ Carotin
a) Oxydationsverlauf im diffusen Tageslicht bei 14° C			
<i>day of preparation</i> Tag der Erzeugung	1,152	1,152	1,152
10. Tag	2,146	1,075	0,693
13. " <i>day</i>	2,146	1,533	1,228
16. "	1,842	6,129	5,514
19. "	1,995	6,280	3,373
b) Oxydationsverlauf im Dunkeln bei 14° C			
<i>day of preparation</i> Tag der Erzeugung	1,152	1,152	1,115
10. Tag	1,152	0,920	0,693
13. " <i>day</i>	0,612	0,462	0,231
16. "	1,075	0,639	0,303
19. "	2,146	0,997	0,770

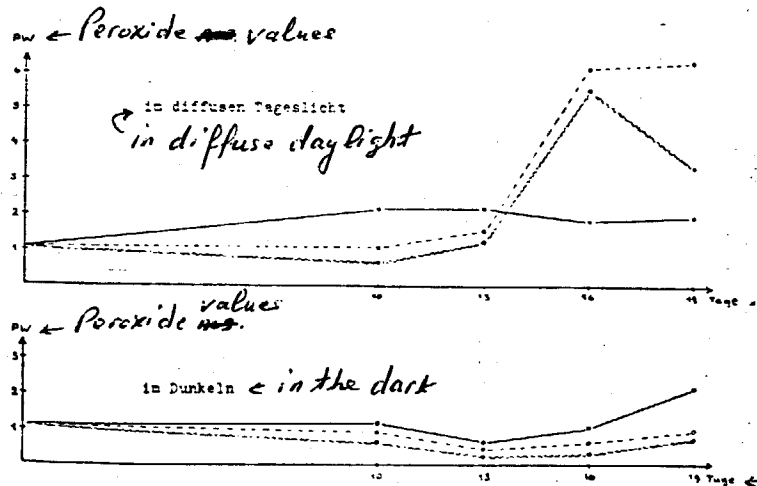


Abb. 4. Oxydationsverlauf von carotinfreier Butter und solcher mit einem Carotingehalt von 8 bzw. 16  $\gamma$ /g. — carotinfrei, - - - 8  $\gamma$  Carotin/g, ..... 16  $\gamma$  Carotin/g

lagen bei 8  $\gamma$ /g — das entspricht etwa dem in der Natur vorkommen- den Höchstgehalt der Butter an Carotin — und bei 16  $\gamma$ /g. Wie aus

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 Tabelle 2. Oxydationsverlauf nach Zugabe von 0,02 mg Ascorbinsäure/100 g Butter

Probenahme am <i>Sampling on</i>	Peroxydwerte <i>Peroxide</i>	
	ohne Carotin	mit Carotin
a) Im diffusen Tageslicht bei 14° C		
<i>day of preparation</i> Tag der Erzeugung	0,539	0,539
6. Tag	1,455	0,770
7. " <i>day</i>	2,376	1,228
9. "	8,425	4,979
b) Aufbewahrt im Dunkeln bei 14° C		
<i>day of preparation</i> Tag der Erzeugung	0,539	0,539
7. Tag	1,151	0,539
9. " <i>day</i>	1,763	0,616
11. "	1,455	2,376

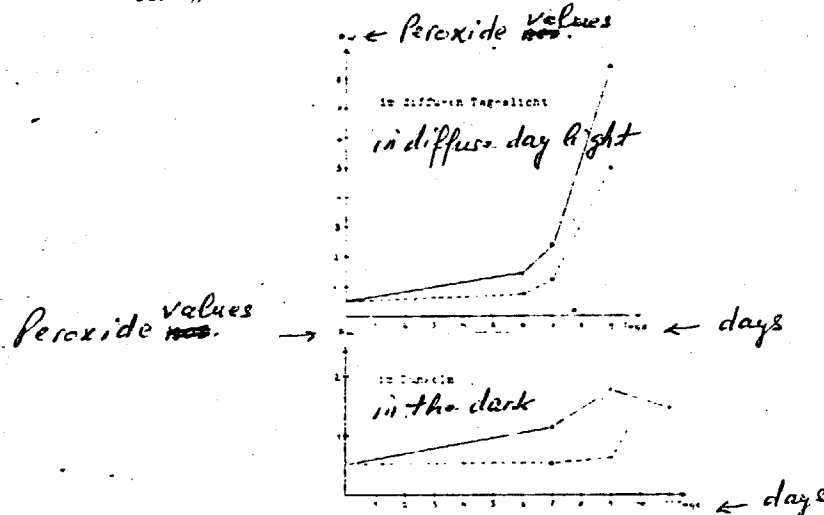


Abb. 5. Oxydationsverlauf nach Zugabe von 0,02 mg Ascorbinsäure/100 g Butter. — carotinfrei, - - - 8  $\gamma$  Carotin/g

Tabelle 1. Abbildung 1. hervorgeht, lagen die Peroxydwerte der carotinhaltigen Butterproben, die dem Tageslicht ausgesetzt waren, bis zum 13. Tag der Lagerung unter denen der carotinlosen Ver-



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gleichsbutter. Nach 16 Tagen Lagerung jedoch überstiegen die Peroxydwerte der carotinhaltigen Proben jene der Vergleichsproben um das Dreifache. Interessanterweise lagen die Peroxydwerte der Butter mit einem Carotingehalt von 16  $\gamma$ /g ständig in fast gleichem Abstand unter den Peroxydwerten der Butter mit einem Carotingehalt von 8  $\gamma$ /g.

Bei den in Dunkelheit aufbewahrten Proben zeigten die carotinlosen bei allen Messungen höhere Peroxydwerte. Bei der carotinhaltigen Butter besaßen die Proben mit einer Konzentration von 8  $\gamma$ /g eine höhere Oxydationsanfälligkeit als jene mit einer Konzentration von 16  $\gamma$ /g.

Anders war der Oxydationsverlauf nach Zugabe von 0,02 mg Ascorbinsäure zu 100 g Butter, dargestellt auf Tabelle 2, Abbildung 5. Diese Butterproben und die der folgenden Versuche waren in Glasröhrchen eingegossen der Oxydation ausgesetzt worden. Die Induktionszeit verkürzte sich auf 6 Tage und in den folgenden Versuchen auf 3 Tage. Die Peroxydwerte der dem Licht ausgesetzten carotinhaltigen Proben lagen auch nach dem jähen Anstieg um 40% unter denen der carotinlosen Vergleichsproben. Bei der Dunkellagerung war eine kleine Verlängerung der Induktionszeit zu beobachten. Die Peroxydwerte der carotinhaltigen Proben, die noch am 9. Tag der Lagerung weniger als die Hälfte jener der Vergleichsproben betrugen, stiegen um den 10. Tag rasch an und lagen am 11. Tag rund 50% über denen der carotinlosen Vergleichsproben. Alle diese Feststellungen ließen eine unmittelbare Mitwirkung des Carotins bei der Butteroxydation erkennen.

Um hierüber genaueren Aufschluß zu erhalten, wurde bei den nächsten Versuchen zugleich mit den Peroxydwerten der jeweilige Carotingehalt bestimmt. Als Zusätze gelangten Kupfer; Diacetyl und Lezithin zur Anwendung; alles Stoffe, die in ihrer Wirkung als Prooxydantien bekannt sind.

## Carotin und Kupfer

Kupfer wurde in einer Konzentration von 0,03 mg/100 g in Form von Kupferlactat zugesetzt. Nach einer kurzen Induktionszeit von 3 Tagen (Tabelle 3, Abbildung 6) stiegen unter Lichteinfluß die Peroxydwerte der carotinhaltigen Proben bis zum 5. Tag jäh an,

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Tabelle 3. Oxydationsverlauf nach Zugabe von 0,03 mg Kupfer pro 100 g Butter

Sampling on	Probenahme am	Peroxydwerte		
		ohne Carotin	mit Carotin	$\gamma$ Carotin, g
a) Ausgesetzt dem diffusen Tageslicht bei 14° C				
→ Tag der Erzeugung	.....	0,539	0,539	8,04
3. Tag	.....	2,350	1,753	5,86
4. " <i>day</i>	.....	4,598	3,982	5,33
5. " <i>day</i>	.....	6,839	6,199	5,23
b) Aufbewahrt im Dunkeln bei 14° C				
→ Tag der Erzeugung	.....	0,539	0,539	8,04
3. Tag	.....	0,612	0,462	6,60
6. " <i>day</i>	.....	1,760	1,380	6,34
9. " <i>day</i>	.....	2,295	2,145	6,33
11. " <i>day</i>	.....	6,585	4,440	6,23

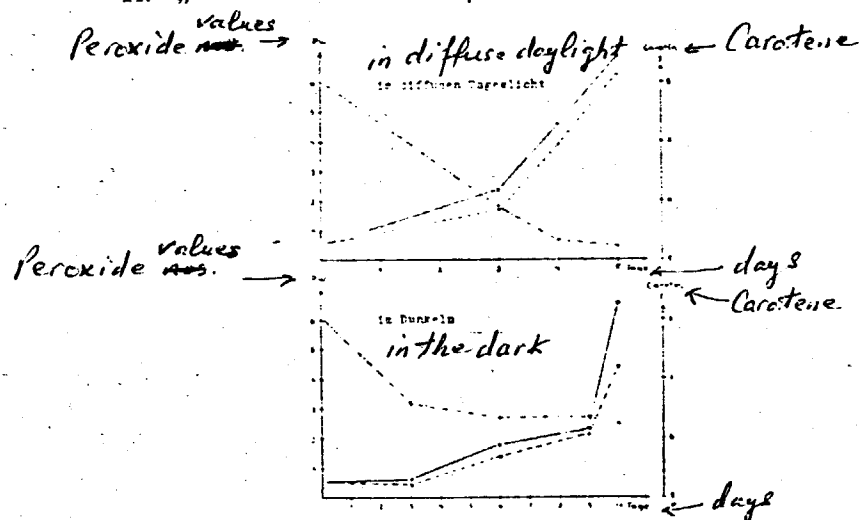


Abb. 6. Oxydationsverlauf nach Zugabe von 0,03 mg Kupfer, 100 g Butter. — carotinfrei, - - - - 8  $\gamma$  Carotin/g, . . . . . jeweiliger Carotingehalt

lagen aber immer um den fast gleichen Betrag von 0,6 Milliäquivalent Sauerstoff unter den Vergleichswerten der carotinlosen

Proben. Der Carotingehalt zeigte bis zum Tag des jähen Oxydationsanstieges einen verhältnismäßig steilen Abfall. Von da an sank er aber nur mehr in geringem Maße.

Die dunkel aufbewahrten Proben zeigten ein ähnliches Bild. Die Induktionszeit verlängerte sich. Die Peroxydwerte der carotinhaltigen Proben übertrafen jene der carotinlosen Proben nie, sondern lagen im Gegenteil an ihrem höchsten Punkt sogar um 2,1 Milliäquivalent Sauerstoff tiefer als die der Vergleichsproben. Der Carotingehalt sank wiederum bis zum Tag der stark ansteigenden Oxydation erheblich, immerhin jedoch nicht in dem Maße wie bei den unter Lichteinfluß gestandenen Proben. Während des steilen Anstieges der Peroxydwerte waren die Verluste nur mehr gering.

#### Carotin und Diacetyl

Nach Zusatz von 0,1 mg Diacetyl/100 g betrug bei den unter Lichteinfluß gestandenen Proben (Tabelle 4, Abbildung 7) die Induktionszeit ebenfalls 3 Tage, wobei wieder ein erhöhter Carotinabbau zu verzeichnen war. Von da an blieb der Carotingehalt fast unverändert. Die Peroxydwerte der carotinhaltigen Proben lagen erst am 5. Tag über denen der Vergleichsproben, und zwar um etwa 0,4 Milliäquivalent Sauerstoff.

Die Induktionszeit bei Dunkelheit dauerte 6 Tage. Erst von hier an überstiegen die Peroxydwerte der carotinhaltigen Proben die der Vergleichsproben und lagen am 10. Tag bereits um das Vierfache darüber. Der Carotingehalt fiel in gleicher Weise wie bei den unter Lichteinfluß gestandenen Proben, nur in einem schwächeren Ausmaße.

#### Carotin und Lecithin

Lecithin wurde in einer Menge von 0,2 g/100 g zugesetzt. Unter Einfluß des diffusen Tageslichtes (Tabelle 5, Abbildung 8) lagen die Peroxydwerte der carotinhaltigen Proben nach der dreitägigen Induktionszeit und während des Oxydationsanstieges um den fast ständig gleichen Betrag von 0,7 Milliäquivalent unter den Vergleichswerten.

Bei Aufbewahrung in Dunkelheit lagen die Peroxydwerte der carotinhaltigen Proben am 3. Tag um 0,8 Milliäquivalent unter

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Tabelle 4. Oxydationsverlauf nach Zugabe von 0,1 mg Diacetyl/100 g Butter

*Samplingen*

Probenahme am	Peroxydwerte		
	ohne Carotin	mit Carotin	$\gamma$ Carotin/g
a) Ausgesetzt dem diffusen Tageslicht bei 14° C			
Tag der Erzeugung	0,539	0,539	8,04
3. Tag	1,610	0,693	6,53
4. " <i>day</i>	3,142	2,911	6,52
5. "	5,210	5,514	6,50
b) Aufbewahrt im Dunkeln bei 14° C			
Tag der Erzeugung	0,539	0,539	8,04
3. Tag	0,235	0,154	7,68
6. " <i>day</i>	0,539	0,462	7,17
9. "	0,997	1,610	7,23
10. "	0,539	2,068	7,16

*without carotene*  
*Peroxide values*  
*with Caroten*  
*carotene*  
*day of preparation*  
*da*

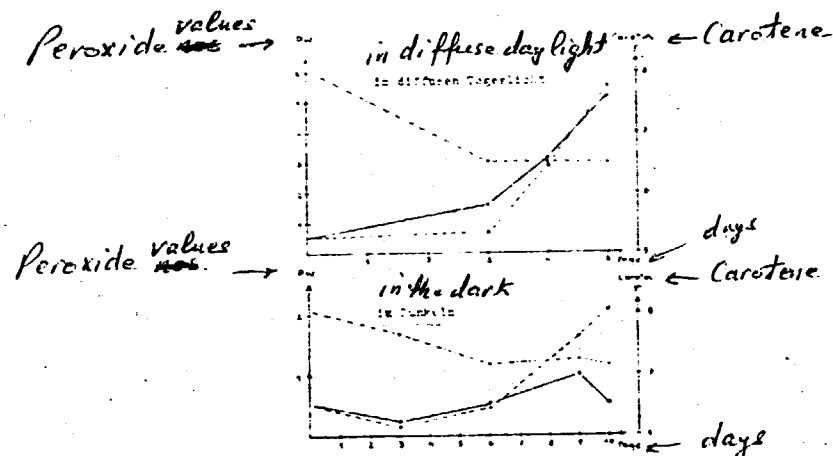


Abb. 7. Oxydationsverlauf nach Zugabe von 0,1 mg Diacetyl/100 g Butter. — carotin frei, - - - 5% Carotin, . . . jeweiliger Carotingehalt

denen der carotinlosen Vergleichsproben, überstiegen diese am 4. Tag und lagen am 5. Tag der Lagerung um 1 Milliäquivalent darüber.

## Besprechung der Ergebnisse

Ein Vergleich der in den Versuchen gewonnenen Kurvenbilder untereinander läßt nun interessante Einzelheiten erkennen. Im großen und ganzen kann man den Oxydationsverlauf nach der Lage der Kurve zueinander in zwei Arten differenzieren:

a) Die Peroxydwerte der carotinhaltigen Proben liegen während des ganzen Oxydationsvorganges unter denen der carotinlosen Vergleichsproben. Letztere besitzen also eine größere Oxydationsanfälligkeit.

Unter Lichteinfluß war diese Erscheinung bei den Proben mit Zusatz von Ascorbinsäure, Kupfer und Lezithin zu erkennen, in Dunkelheit jedoch nur bei solchen mit Kupferzusatz.

b) Die Peroxydwerte der carotinhaltigen Proben übertreffen jene der carotinlosen Vergleichsproben erst nach Ablauf einer gewissen Zeitspanne.

Diesen Verlauf zeigten unter Lichteinfluß die Proben mit Zusatz von Diacetyl, bei Aufbewahrung in Dunkelheit jene mit Zusatz von Ascorbinsäure, Kupfer und Lezithin.

Reinart (27) verwendete bei seinen Versuchen in Glasröhrchen eingeschmolzenes Butterfett und setzte dieses bei Zimmertemperatur der Einwirkung von diffusem Tageslicht aus. Die zugesetzte Menge an Carotin betrug 0,02%. Die verwendete Methode der Peroxydbestimmung nach Lea besitzt aber nicht die gleiche Empfindlichkeit wie die Ferri-thiocyanatmethode. Trotzdem lagen auch hier die Peroxydzahlen der carotinhaltigen Proben bis zum 10. Tag der Lagerung unter den Peroxydzahlen der Vergleichsproben und überstiegen diese erst nach dem 15. Tag. Eine ähnliche Übereinstimmung mit den eigenen Untersuchungen liegt bei den Proben mit Diacetylzusatz vor, obwohl die verwendete Menge Diacetyl den natürlich vorkommenden Gehalt bedeutend übersteigt. Auch hier war bei den carotinhaltigen Proben anfangs eine geringere Oxydigkeit festzustellen, die aber nach 25 Tagen Lagerung jene der carotinlosen Vergleichsproben übertraf.

Eine Übereinstimmung mit den Ergebnissen des eigenen Versuches zeigt sich auch bei diacetylhaltiger Butter, die in Dunkelheit aufbewahrt worden war.

Lediglich in der Zeit ergeben sich Unterschiede. Diese können auf die Art der Behandlung des Fettes, aber auch auf die Art der Aufbewahrung zurückzuführen sein. (Reinart benützte reines Butterfett ohne Wasserezusatz, während die eigenen Untersuchungen an Butter, die einen Wassergehalt von 18% aufwies, angestellt wurden.)

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Tabelle 5. Oxydationsverlauf nach Zugabe von 0,2 g Lezithin pro 100 g Butter

Probennahme am Sampling on	Peroxydwerte		
	ohne Carotin	mit Carotin	γ Carotin g
a) Ausgesetzt dem diffusen Tageslicht bei 14° C			
Tag der Erzeugung	0,539	0,539	8,04
3. Tag	1,841	1,074	6,64
4. " day	3,677	2,988	6,22
5. " "	5,822	5,056	6,04
b) Aufbewahrt im Dunkeln bei 14° C			
Tag der Erzeugung	0,539	0,539	8,04
3. Tag	1,305	0,539	6,84
4. " day	1,918	2,149	6,67
5. " "	3,219	4,289	6,50

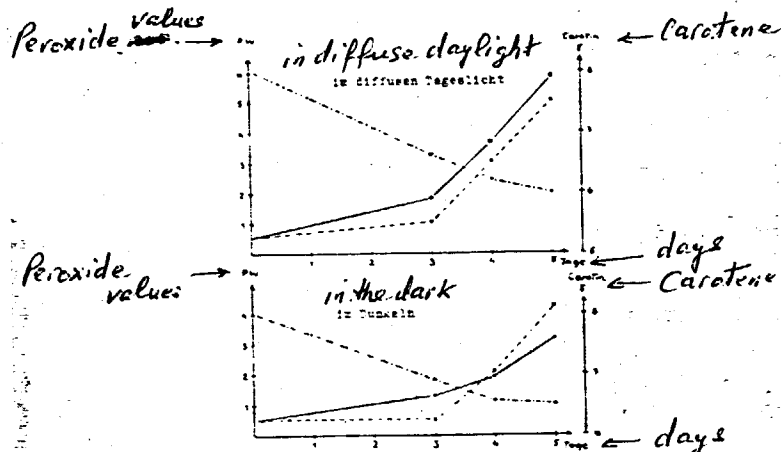


Abb. 5. Oxydationsverlauf nach Zugabe von 0,2 g Lezithin/100 g Butter. — carotin-frei, - - - - - 0,2 γ Carotin g, . . . . . jeweiliger Carotingehalt

Der Carotinabbau war in beiden Fällen während der Induktionszeit umfangreich, verlief jedoch nach Anstieg der Peroxydwerte wesentlich langsamer.

Thompson und Steenbock (33) verwendeten für ihre Untersuchungen das Warburg-Respirometer. In diesem Gerät wird eine bestimmte Menge Fett bei bestimmter Temperatur dem Sauerstoffdruck ausgesetzt. Der manometrisch gemessene Druckabfall gilt als Maß für die Sauerstoffaufnahme des in Oxydation begriffenen Fettes. Die in diesem Falle verwendete Versuchsmenge betrug 1 g mit einem Carotinzusatz von 250  $\gamma$ , das sind 0,025%, während der Höchstgehalt im Milchl-fett nur etwa 0,0008% beträgt.

Aus dem erhöhten Sauerstoffverbrauch der carotinhaltigen Proben schlossen sie auf eine höhere Fettoxydierbarkeit dieser Proben. Die gewonnenen Ergebnisse besagen aber lediglich, daß Fett mit einem Carotinhalt von 0,025% mehr Sauerstoff aufnimmt als carotinloses Fett. Offen geblieben ist aber die Frage, wieviel von dem aufgenommenen Sauerstoff zum Carotinabbau nötig war, zumal ein solcher bei der Betriebstemperatur des Warburg-Respirometers von 37° C und unter dem herrschenden Sauerstoffdruck auch unabhängig von der Fettoxydation für durchaus möglich gehalten werden muß. Ein klareres Bild hätte sich möglicherweise ergeben, wenn neben der Sauerstoffaufnahme des zu untersuchenden Gutes noch der gleichzeitige Carotinabbau festgestellt worden wäre.

Das Warburg-Respirometer fand auch bei den Versuchen von Schulte und Schillinger (30) Verwendung, deren Ziel die Kenntnis der gekuppelten Autoxydation von Fetten mit Vitamin A und dessen Provitamin, dem  $\beta$ -Carotin war. Die Vitaminkonzentration belief sich auf  $15 \cdot 10^{-6}$  Mol in 2 ml Ester. Aus den Ergebnissen geht ebenfalls eine erhöhte Sauerstoffaufnahme der carotinhaltigen Proben hervor. Die von ihnen festgestellten Werte würden möglicherweise auch einen Vergleich zwischen den stattgefundenen Vorgängen des Fettabbaues auf Grund des Ausmaßes des Vitaminabbaues ermitteln lassen. Dieser Berechnung würde aber eine gewisse Unsicherheit anhaften, da es noch keine exakte Angabe über das stöchiometrische Verhältnis gibt, nach welchem Vitamin A und Sauerstoff reagieren und gewisse am Sauerstoffverbrauch beteiligte Sekundärreaktionen angenommen werden müssen.

Einen interessanten Einblick bietet aber folgende Darstellung:

Sauerstoffaufnahme und Vitamin-A-Acetat-Abnahme einer Lösung von Vitamin-A-Acetat in einem Gemisch aus Laurinsäureester und Ölsäureester (1:1) bei verschiedenen Vitamin-A-Konzentrationen nach 6 Stunden

Vitamin-A-Konzentration in Mol/Ansatz	O <sub>2</sub> -Aufnahme in $\mu$ l	Vitamin-A-Acetat- Abnahme in %
$7,5 \cdot 10^{-6}$	238	28
$15 \cdot 10^{-6}$	258	30
$30 \cdot 10^{-6}$	280	29

Table

Vitamin A concentration  
in moles/batch

O<sub>2</sub>-uptake  
in microliters

Vitamin A  
acetate  
decrease

Die perzentuellen Vitamin-Verluste sind in den drei Fällen gleich. Umgerechnet auf die einzelnen Vitamin-Konzentrationen würden diese Mengen aber  $2,1 \cdot 10^{-6}$ ,  $4,5 \cdot 10^{-6}$  und  $8,7 \cdot 10^{-6}$  Mol/Ansatz betragen. Dieser progressiven Vitamin-A-Abnahme steht aber nur eine linear erhöhte Sauerstoffaufnahme gegenüber. Es könnte angenommen werden, daß der für die Vitamin-A-Oxydation sich ergebende Sauerstoffbedarf durch die weitere Aufnahme von etwa 20  $\mu$ l nicht zur Gänze gedeckt werden kann und aus diesem Grunde weniger Sauerstoff für die Fettoxydation verfügbar ist. Ähnliche Beobachtungen wurden an eigenen Versuchen (siehe Abbildung 4) gemacht, wo die Peroxydwerte der Proben mit einer Carotin-Konzentration von 16  $\gamma$ /g Butter immer unter den Peroxydwerten der Proben mit einem Gehalt von 8  $\gamma$ /g Butter lagen.

Eine wertvolle Vergleichsmöglichkeit brachten die in den eigenen Untersuchungen zugleich mit der Peroxydmesung durchgeführten Carotinbestimmungen mit den bereits erwähnten Ergebnissen von Holmann (10).

Die Untersuchungen der vorliegenden Arbeit bezogen sich nur auf die Bestimmung der ersten Oxydationsanzeichen, der Peroxyde. Aber auch in diesem Stadium konnten bereits erhebliche Carotin-Verluste festgestellt werden. Diese lagen bei Proben, die unter Einfluß des Tageslichtes gestanden waren, zwischen 20 und 35%, bei solchen, die in Dunkelheit aufbewahrt worden waren, zwischen 11 und 23%. In allen Fällen stiegen die Peroxydwerte aber erst an, nachdem der Carotin-Gehalt relativ stark abgenommen hatte.

Diemayr und Mitarbeiter (5) verwendeten bei ihren Bestimmungen Modellester. Davon wurden 5 cm<sup>3</sup> mit 2,5 mg  $\beta$ -Carotin bei 94° C mit Sauerstoff belüftet. Es wäre zufolge der hohen Temperatur und des starken Sauerstoffeinflusses durchaus möglich, daß in bezug auf die Peroxydbildung nach 30 und 60 Min. bereits ein ähnlicher Zustand eingetreten ist, wie er bei den eigenen im Tageslicht anzutreffenden Proben (Abbildung 4) erst am 16. Tag der Lagerung anzutreffen war. Die Bestimmung der Oxydierbarkeit erfolgte durch die Ermittlung der Peroxydzahl nach Lea, Walser (35) zufolge ist aber dieser Methode eine gewisse Subjektivität nicht abzuspüren. Ebenso besitzt sie, wie bereits erwähnt, nicht die erforderliche Empfindlichkeit.

Nach den Ergebnissen von Hinko (9) ging mit der Mehraufnahme von Sauerstoff im Fett bei Anwesenheit von Carotin gleichzeitig ein Carotinabbau vor sich. Er berichtete aber anschließend von praktischen

Erfahrungen, die gezeigt haben, daß Carotin bei gelagerten Fetten einen gewissen Oxydationsschutz darstelle.

Eine interessante Vergleichsmöglichkeit lieferte die Arbeit von Chevalier (4), der, wie vorne berichtet, bei Lichteinfluß eine prooxydante und in Dunkelheit eine antioxydante Wirkung des Carotins feststellte. Diese Ergebnisse kamen zustande, nachdem 10 mg Triolein in dünner Schicht bei 40° C der Oxydation ausgesetzt worden waren. Ein Teil der Proben enthielt 50 bis 80  $\gamma$   $\beta$ -Carotin. Die Lichteinwirkung wurde durch eine Quecksilberdampflampe, mit Filter begrenzt auf eine Wellenlänge von 3400 Å, erreicht. Die in Dunkelheit aufbewahrten Proben zeigten folgende Ergebnisse:

Stunden	1	2	3	4	5	10	15	17
Triol. + Car.	0,87	0,79	0,76	0,71	0,68	0,71	0,74	0,80
Triolein								

Der Oxydationsquotient der in Dunkelheit aufbewahrten Proben zeigt die antioxydante Wirkung des Carotins, die aber mit zunehmender Zeitdauer (ähnlich wie auf Abbildung 4) abnimmt. Die Oxydationsquotienten der unter Lichteinfluß gestandenen Proben hingegen waren:

Stunden	1	2	3	4	5	6	7	8
Triol. + Car.	1	1,17	1,25	1,28	1,30	1,29	1,19	1,26
Triolein								

Es ist anzunehmen, daß als Folge der sehr intensiven Bestrahlung die Oxydationswerte der carotinhaltigen Proben bereits nach einer Stunde auf gleicher Höhe mit denen der carotinfreien Vergleichsproben stehen, um dann weiter anzusteigen, ein Zustand, der bei den unter Einfluß des diffusen Tageslichtes bei einer weit niedrigeren Temperatur gestandenen Proben der eigenen Versuche (Abbildung 4) erst am 14. Tage der Lagerung eintritt.

### Zusammenfassung

1. Zur Feststellung, ob oder wie weit dem  $\beta$ -Carotin bei der Butteroxydation eine Rolle zukommt, wurden vergleichende Untersuchungen an Modellbutter, mit und ohne Carotin, bzw. mit und ohne verschiedene Zusätze durchgeführt.

2. Bei Butter ohne jeglichen prooxygenen Zusatz zeigte sich während der Lagerung bei etwa 14° C in diffusen Tageslicht eine oxydationshemmende Wirkung des Carotins bis zum 13. Tag. Erst von hier an beteiligte sich das Carotin prooxydant. Im Dunkeln war die antioxydante Wirkung des Carotins die ganze Untersuchungsperiode von 19 Tagen hindurch erkennbar.

Bei Verwendung von Proben mit verschiedenen Carotin-Konzentrationen besaßen jene mit niedrigerem Carotin-Gehalt eine höhere Oxydationsanfälligkeit.

3. Während der Induktionszeit lagen bei allen Proben die Peroxydwerte der carotinhaltigen Proben unter denen der carotinfreien Vergleichsproben.

4. Eine durchwegs oxydationshemmende Wirkung des Carotins bei Tageslicht zeigten Proben, denen Ascorbinsäure, Lecithin oder Kupfer zugesetzt worden waren.

Im Dunkeln war eine solche oxydationshemmende Wirkung des Carotins nur bei den Proben mit Kupferzusatz festzustellen.

5. Eine anfänglich oxydationshemmende, später aber oxydationsfördernde Wirkung des Carotins war an Butterproben mit Zusatz von Diacetyl sowohl bei Aufbewahrung im Tageslicht wie auch im Dunkeln zu erkennen; Proben mit Zusatz von Ascorbinsäure oder Lecithin zeigten nur im Dunkeln ähnliches Verhalten.

6. Der Carotinabbau war während der Induktionszeit heftig, verringerte sich aber bei fortschreitender Fettoxydation.

7. Es wäre daraus zu schließen, daß das Carotin während der Induktionszeit einen Schutzfaktor für das Fett darstellt, derart, daß es durch Auffangen des von Lichtenergie oder verschiedenen Prooxydanten aktivierten Sauerstoffes das Fett vor oxydativem Angriff schützt, selbst aber dabei zum Teil abgebaut wird. Möglicherweise schalten sich erst die gebildeten Abbauprodukte als Oxydationsfaktoren in die weiteren Vorgänge des Fettverderbens ein.

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# Taste Thresholds of Butter Volatiles in Deodorized Butteroil Medium

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**SUMMARY**—Taste thresholds of 31 volatile compounds found in butter were measured in deodorized butteroil and thresholds of seven volatiles were measured in fresh butter. Thresholds of mixtures of each of the major classes of volatile compounds (free fatty acids from  $C_2$  through  $C_{12}$ , gamma-lactones from  $C_7$  through  $C_{11}$ , even-numbered delta-lactones from  $C_8$  through  $C_{12}$  and methyl ketones from  $C_3$  through  $C_{13}$  except  $C_{12}$  were determined as well as thresholds of single compounds of these classes. Butyric acid, diacetyl, delta-decalactone, 2-nonanone, gamma-undecalactone and n-hexanal, oft-reported constituents of milk fat, had thresholds in butteroil of 0.66, 0.055, 1.4, 7.7, 0.95 and 0.19 ppm, respectively. The threshold of a mixture of free fatty acids from  $C_2$  through  $C_{12}$  was 0.55 ppm. Synergistic interactions among methyl ketones and free fatty acids were pronounced and interactions among aldehydes were weak, while interactions among lactones were not apparent.

## INTRODUCTION

MORE THAN 100 volatile compounds have been identified as natural constituents of butter or milk fat (Day et al., 1960; Forss et al., 1967; Jurriens et al., 1965; Langler et al., 1964, and Wong, 1963). A small number of these compounds are generally recognized as principal components of butter flavor. Different types of butter flavor concentrates are available from at least 41 commercial suppliers. None of these concentrates duplicates the complete natural flavor of butter, nor are all the compounds in these concentrates necessarily natural butter aroma constituents.

Human taste and odor thresholds of individual compounds are indexes of flavor (Patton et al. 1957). Thresholds of mixtures of compounds have been shown to complicate interpretations of flavor chemistry due to additive, synergistic and antagonistic interactions of mixtures (Day et al. 1963; Langler et al. 1964; Meijboom, 1964). Water thresholds of many butter volatiles are known. To complement the water threshold data, taste thresholds of key butter volatiles in butteroil medium and in butter itself were needed.

This study was undertaken to determine butteroil thresholds of individual compounds as well as mixtures of the prominent classes of volatile compounds found in butter. Compounds included were free fatty acids (even-numbered,  $C_2$  through  $C_{12}$ ), delta-lactones ( $C_8$ ,  $C_{10}$ ,  $C_{12}$ , and  $C_{14}$ ), gamma-lactones ( $C_7$  through  $C_{11}$ ), methyl ketones ( $C_3$  through  $C_{11}$ ,  $C_{13}$ , and  $C_{15}$ ) and selected miscellaneous compounds which are known to be present in butter.

## EXPERIMENTAL METHODS

### Preparation of odor-free butteroil

Butteroil separated from fresh, melted sweet cream butter was filtered through Eaton & Dikeman No. 17 filter paper to remove the remaining free butter serum. The filtered oil was then vacuum steam-distilled in an all-glass apparatus designed to handle 10 to 12 L. of oil (Fig. 1). Earlier studies revealed that relatively high temperatures were required to remove butter flavor from the oil. In a typical vacuum steam distillation, melted oil was heated to  $210^\circ \pm 10^\circ\text{C}$ , held at this temperature for about 2 hr. then allowed to cool to  $140^\circ\text{C}$ . Antioxidant was added according to the method of Wyatt et al. (1965). The pressure above the oil, which increased with temperature, ranged from 9 to 23 mm (Hg), and was 1 mm or less at the pump end of the system. The amount of water distilled was  $\frac{1}{4}$  to  $\frac{1}{2}$  the oil volume.

Volatile compounds, as well as cholesterol and carotenoids, were removed by the distillation. The deodorized oil had a faint odor and a slightly sweet or nutty taste. A recog-

nizable butter flavor, however, was not detectable.

### Purification of compounds tested

For flavor studies, reagent grade chemicals whose normal boiling points were below  $230^\circ\text{C}$  were vacuum-distilled in an all-glass 10 to 50-ml capacity distillation apparatus. Lauric, myristic, palmitic and oleic acids were purified by several recrystallizations from ethanol-water. Other high boiling compounds were purified by preparative GLC, using a  $\frac{1}{4}$  inch  $\times$  4.5 ft aluminum column containing 80/120 mesh glass beads coated with 0.1% Apiezon H. Purity of the liquids, as determined by gas chromatography (150 feet  $\times$  0.01 in. I.D. stainless steel capillary coated with Ucon), was better than 99.8% except for 2-pentanone (99.6%).

### Testing procedures

Glassware used in all tests was thoroughly cleaned and carefully inspected to exclude containers which had residual stains or odors. Stock solutions were prepared in 100-ml volumetric flasks by dissolving calculated amounts of purified compounds in deodorized oil at  $38^\circ \pm 4^\circ\text{C}$  and bringing to volume; four or five dilutions to lower concentrations were made from each stock solution. Compounds whose thresholds were measured in water were brought into solution by first dissolving the compound in ethanol and then dispersing the ethanol solution into water (subthreshold ethanol concentration was maintained).

The flavor tests were conducted by the procedure described by Wyatt et al. (1965).

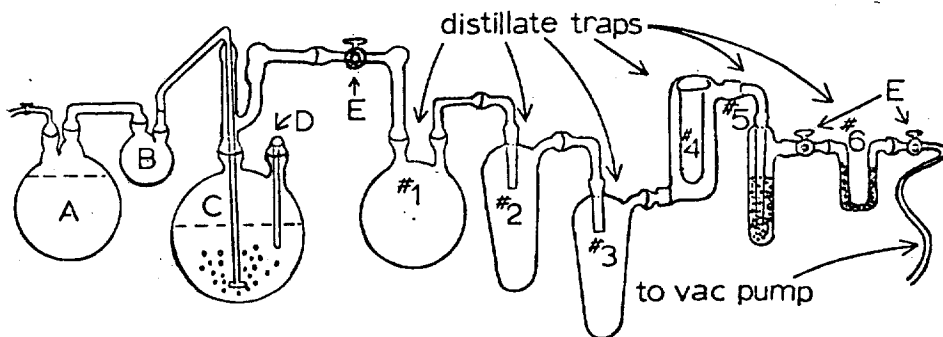


Fig. 1—Vacuum steam distillation apparatus used. A—steam reservoir, B—safety trap, C—substrate receptacle, D—thermometer well, E—stockcocks; traps #1 and #2—dry ice-acetone cooled; traps #3 through #6—liquid nitrogen cooled traps (traps #5 and #6 contain glass beads).

Table 1—Taste thresholds of selected butter volatiles measured in deodorized butteroil

Compound	Concentration in ppm	
	Threshold	Approximate level in fresh butter <sup>1</sup>
Butyric acid	0.66	9 to 38
Caproic acid	2.5 <sup>2</sup>	6 to 18
Diacetyl	0.055	0.02 to 0.1
Dimethyl sulfide	0.009	0.02
Delta-decalactone	1.4	0.01 to 9
Ethyl acetate	22	4
Ethyl butyrate	0.60	<0.2
Ethyl hexanoate	0.85	<0.1
Acetaldehyde	0.11	0.7
<i>n</i> -Hexanal	0.19	0.14
<i>n</i> -Heptanal	0.75	0.16
<i>n</i> -Nonanal	1.0 <sup>3</sup>	0.07

<sup>1</sup> Concentration values were from the following sources: fatty acids, Iyer et al. (1967); aldehydes, Day et al. (1960); and delta-decalactone by Forss et al. (1967) (0.01) and Jurriens et al. (1965). Other concentrations were estimated from GLC analysis of butter volatiles (unpublished data, T. J. Siek).

<sup>2</sup> From Patton (1964).

<sup>3</sup> From Hvolby (1962).

Four or five dilutions of the compound being tested were served to tasters along with a labeled blank (zero concentration) and a coded blank. The panel of judges consisted of 28 members of the Food Science and Technology staff with 20 members used per test; oil samples were served at  $42^\circ \pm 3^\circ\text{C}$ ; and judges were not pre-selected for taste acuity, but were experienced in serving on flavor panels.

In the flavor booths, judges were asked to taste the two reference samples (zero concentration and the highest concentration) and then to taste at random the coded samples containing the flavor compound. The judges marked their ballot "plus" when the compound in question was detected in a sample. No time limit was imposed during tasting. If panel members misjudged the coded blank and/or coded maximum concentration sample, their ballot was excluded from the results. Otherwise, each "plus" answer was recorded. Taste tests were conducted within a week after the sample purification. Solutions of compounds tested were made on the day of the test. The 50% posi-

Table 3—Taste thresholds of methyl ketones in deodorized butteroil—individual compounds and mixtures

Carbon number	Concentration in ppm	
	Individual threshold <sup>1</sup>	Ketone in mixture at mixture threshold
C <sub>3</sub>	125	0.09
C <sub>4</sub>	30	0.09
C <sub>5</sub>	61	2.6
C <sub>6</sub>	—	0.09
C <sub>7</sub>	15	3.5
C <sub>8</sub>	2.5	0.09
C <sub>9</sub>	7.7	2.6
C <sub>10</sub>	11	0.09
C <sub>11</sub>	100	5.3
C <sub>12</sub>	182	3.5
C <sub>13</sub>	—	5.3
C <sub>3</sub> to C <sub>13</sub>	23 = mixture threshold	

<sup>1</sup> Values obtained by Hvolby (1962) for C<sub>4</sub>, C<sub>5</sub>, C<sub>10</sub>, C<sub>11</sub>, and C<sub>12</sub> were 30, 5, 10, 10, and 500 respectively.

Table 2—Taste thresholds of free fatty acids in deodorized butteroil—individual compounds and mixtures

Fatty acid	Individual threshold <sup>1</sup>	Concentrations in ppm	
		Fatty acid in mixture at the mixture threshold <sup>2</sup>	
		I	II
C <sub>2</sub>	7.0	0.002	0.003
C <sub>3</sub>	0.66	0.07	0.10
C <sub>4</sub>	2.5	0.03	0.06
C <sub>5</sub>	350	0.05	0.08
C <sub>10</sub>	200	0.11	0.17
C <sub>12</sub>	700	0.29	0.45
C <sub>2</sub> -C <sub>12</sub>	1260	0.55 = mixture threshold	
C <sub>14</sub>	5000		814
C <sub>16</sub>	10000		2203
C <sub>18</sub>	15000		617
C <sub>18:1</sub>	8000		1315
C <sub>2</sub> -C <sub>18:1</sub>	39260	5000 = mixture threshold	

<sup>1</sup> Individual thresholds were taken from Feron et al. (1961) for C<sub>6</sub> through C<sub>18:1</sub>; their value for C<sub>4</sub> was 0.60.

<sup>2</sup> Column I was a mixture through C<sub>12</sub>; column II contained all fatty acids listed.

tive response level used by Patton et al. (1957) was calculated, so that direct comparisons might be made with reported 50% thresholds. Threshold concentrations are defined as concentrations of compounds in a given medium in parts per million.

For comparison, several thresholds were measured in fresh sweet cream butter. The butter samples were served from the refrigerator ( $4^\circ\text{C}$ ) as butter patties. Mixture solutions of several classes of butter volatiles were also prepared for testing. Mixture thresholds were established in the same manner as single-compound thresholds, the mixture threshold being the total volatile concentration which could be detected. Ratios of individual compounds were constant in each dilution of the mixture.

Threshold determinations usually required one or more preliminary tests to find the appropriate concentration range. In practice, panelists often sniffed all samples and tasted only those they could not categorize by sniffing. Panelists could usually detect one or two concentrations lower by tasting than they could by sniffing only. Meijboom (1964) found that for 31 aldehydes, threshold values for taste (measured in paraffin oil) were in all cases lower than those for odor.

1965, p. 182); thus differences in taste thresholds from different laboratories are probably due to differences in make-up of the panel. By observing general trends of a homologous series (Table 3) more validity can be ascribed to individual threshold values, and from these values a measure of relative flavor potential can be ascertained.

Table 4—Taste thresholds of normal aldehydes in deodorized butteroil—individual compounds and mixture

Carbon number	Concentration in ppm	
	Individual threshold	Aldehyde in mixture at mixture threshold
C <sub>5</sub>	0.30	0.16
C <sub>6</sub>	0.19	0.16
C <sub>7</sub>	0.75	0.16
C <sub>8</sub>	0.9 <sup>1</sup>	0.16
		0.64 = mixture threshold

<sup>1</sup> From Lea and Swoboda (1958); their value for *n*-hexanal was 0.3.

## RESULTS & DISCUSSION

### Individual thresholds

Taste thresholds of several butter compounds are presented in Table 1 along with their reported or estimated concentrations in butter. Other individual thresholds are shown in Tables 2, 3, 4, and 5.

The threshold of delta-decalactone was measured five times (on five days) with some variation of panel members in each test. The threshold obtained was  $1.4 \pm 1.1$  ( $\sigma$ ). Greater precision was noted in repeated tests with other compounds: for 2-pentanone the threshold was  $61 \pm 3$ ; gamma-undecalactone,  $0.95 \pm 0.22$ , and *n*-hexanal,  $0.016 \pm 0.003$  (in water). Thresholds depend greatly on expertise

Table 5—Taste thresholds of lactones in deodorized butteroil—individual compounds and mixtures

Lactone	Concentration in ppm	
	Individual threshold	Lactone in mixture at mixture threshold <sup>1</sup>
$\gamma$ -C <sub>5</sub>	8.0	—
$\gamma$ -C <sub>7</sub>	3.4	0.47
$\gamma$ -C <sub>8</sub>	3.5	0.94
$\gamma$ -C <sub>9</sub>	2.4	0.94
$\gamma$ -C <sub>10</sub>	1.0	0.94
$\gamma$ -C <sub>11</sub>	0.95	0.94
		4.3 = mixture threshold
$\delta$ -C <sub>8</sub>	3.0	0.54
$\delta$ -C <sub>10</sub>	1.4	2.7
$\delta$ -C <sub>12</sub>	95	5.4
$\delta$ -C <sub>14</sub>	500	5.4
		14 = mixture threshold

<sup>1</sup> Gamma- and delta-decalactone mixtures were



### Thresholds of mixtures

Thresholds of individual compounds and thresholds of mixtures of several classes of butter volatiles are given in Tables 2, 3, 4 and 5 (note that in Tables 2 to 5, individual compound concentrations are given above the mixture threshold concentration and individual thresholds are in a separate column). Methyl ketone, free fatty acid and aldehyde homologous series mixtures exhibit results similar to those reported by Langer et al. (1964). Interaction in the mixtures is apparent especially with fatty acid and methyl ketone mixtures (Tables 2 and 3), as at the mixture threshold, concentrations of individual compounds are sub-threshold. Langer et al. (1964) refer to such effects as "synergistic." The synergistic effect was not pronounced with aldehydes and was not evident among lactones.

Table 6 gives the water, oil and butter taste thresholds of several selected compounds obtained in this study and by other investigators. The data show that oil thresholds are generally higher than water thresholds. Thresholds in butter are closer to oil thresholds than water thresholds. Factors influencing water and oil threshold differences have been discussed previ-

ously (Lea et al. 1958; Patton 1964).

### CONCLUSIONS

Among the thresholds measured, those of several butter volatiles are lower (or nearly so) than their reported concentration in butter, and thus would be expected to contribute to sweet cream butter flavor. Compounds that fall into this group are diacetyl, butyric and caproic acids, hexanal, acetaldehyde, dimethyl sulfide and possibly delta-decalactone. Levels of 2-heptanone and 2-nonanone (Langer et al. 1964) could be high enough to influence flavor due to synergistic interactions. The aroma and flavor of fresh cream butter depend on a concentration balance of low threshold compounds reported herein (plus possibly some volatiles not as yet identified in butter), with little contribution to flavor by high threshold compounds. Synergistic interactions exhibited by mixtures probably play an important role in giving butter its unique flavor and aroma.

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Table 6—Taste thresholds of representative volatile compounds in different media

Compound	Threshold concentration in ppm			
	Water	Oil	Milk	Butter
Ethyl acetate	6.6	22	4.7	—
Ethyl butyrate	0.015	0.60	0.016	—
Dimethyl sulfide	—	0.009	0.019 <sup>1</sup>	0.17
Diacetyl	0.0054	0.055	0.014	0.032
2-Octanone	0.15	2.5	—	3.4
2-Decanone	0.19	11	—	9.3
Delta-decalactone	0.14	1.4	—	—
Acetic acid	22	7.0	25 <sup>2</sup>	—
Butyric acid	6.2	0.66	14	—
Caproic acid	15	2.5 <sup>2</sup>	23 <sup>2</sup>	—
Caprylic acid	5.3 <sup>3</sup>	350 <sup>2</sup>	28 <sup>2</sup>	—
Capric acid	3.5 <sup>3</sup>	200 <sup>2</sup>	—	10
n-Heptanol <sup>4</sup>	2.4	20	—	—
n-Dodecanal <sup>4</sup>	0.011	0.75 <sup>5</sup>	—	—
Acetaldehyde	1.3 <sup>4</sup>	0.11	0.13 <sup>7</sup>	—
n-Pentanal	0.07	0.30	0.05 <sup>7</sup>	0.80
n-Hexanal	0.016	0.19	0.12 <sup>7</sup>	0.90
n-Heptanal	0.031	0.75	—	—

<sup>1</sup> From Reddy et al. (1967).

<sup>2</sup> From Patton (1964); his values for acetic, butyric and caproic acids were 54, 6.8 and 5.4 respectively.

<sup>3</sup> Concentration that gives a rancid flavor (Scanlan et al. 1965); butyric and caproic acid values (rancid flavor) were 46 and 30 respectively.

<sup>4</sup> Not indigenous to butter.

<sup>5</sup> Lea et al. (1958) reported 0.9.

<sup>6</sup> From Berg et al. (1955).

<sup>7</sup> From Day et al. (1963).

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# 2,3-Butanedione, an electron-stabilizing compound, as a modifier of sensitivity of *Bacillus megaterium* spores to x-rays†

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The organic compound 2,3-butanedione (diacetyl) has a high affinity for free electrons, and can stabilize electrons produced by high-energy radiations in aqueous solutions. In the non-metabolizing spore of *Bacillus megaterium* (butler suspension at pH 7.0), this compound increases radiation sensitivity in the absence of oxygen as concentration increases up to approximately  $4 \times 10^{-3}$  M. The maximal increase represents 40 per cent of the total observed in the full oxygen effect. Since the compound at maximally effective concentration in the presence of oxygen does not increase radiation sensitivity above that level seen in oxygen alone, it is allowed that the potentiating action of the compound represents one of the actions of oxygen, i.e. oxygen may act in part by stabilizing electrons. Above about  $6 \times 10^{-4}$  M the effectiveness of the compound as a sensitizer decreases, until the anoxic level of sensitivity is reached at  $6 \times 10^{-2}$  M. No suggestions concerning this reversal of effect at high concentrations are offered at this time.

## Introduction

The mechanism(s) of the action of oxygen in increasing the sensitivity of stabilizing cells to x-irradiation has not been elucidated experimentally. One set of experiments on certain chemical compounds that sensitize cells irradiated in the absence of oxygen can be used as a basis for a proposition concerning the nature of one of these mechanisms. Adams and Dewey (1963) have shown that certain compounds with high electron affinities and properties conducive to stabilization of the attached electron do sensitize *Serratia marcescens* to x-irradiation, and almost to the degree seen with oxygen. This reminds us again of the suggestion made by several authors in the past that oxygen can be functioning (at least in part) as an electron acceptor in effecting its sensitizing action.

Because of its biochemical inertness, the bacterial spore is a very suitable biological object for testing this proposal in the absence of ordinary biochemical activities involving oxygen that confound interpretations of results with metabolizing cells. Furthermore, the absence of toxicity of one of these compounds at concentrations that show radiobiological activity in the spore (see below) signifies an element that confuses interpretation in some other biological terms. In this paper, we report the results of tests of this compound.

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## 2. Materials and methods

2,3-Butanedione, research grade, was used as supplied by Matheson, Coleman and Bell.

The test organism was the spore of *Bacillus megaterium* (ATCC No. 8245), produced as reported earlier (Powers, Ehret and Bannon 1957) and stored at 4°C in distilled water. The plating medium was double-strength MRVP medium (Difco) solidified by 1.5 per cent agar. All dilutions of spore suspensions after irradiation were done in phosphate buffer (pH 7.0) to give numbers of colonies between 30 and 200 per plate.

X-irradiation was carried out with spores suspended in 15 mM phosphate buffer at pH 7.0. The x-ray source was a Machlett OEG 60 tube operated at 50 kVp and 37.5 mA (with 0.002 in. Al filter), powered by a Philips constan-t-potential generator. The dose-rate was 9.4 krad/min as determined by the ferrous sulphate dosimeter in a geometry identical to that in spore experiments. It was assumed that  $G(\text{Fe}^{3+}) = 14.0$  (Back and Miller 1957).

For irradiation of spores in anoxia, 3 ml. of a mixture of spores and diacetyl at desired concentrations in buffer were placed into each of several vacuum-tight vessels; the depth of liquid in each vessel was 0.5 cm. Dissolved air was removed from the suspension by repeated expansion of the vessels' atmospheres into a high-vacuum manifold about 20 times greater in volume than a vessel, with concurrent stirring of the liquid phase. The pressure in the manifold at the start of each expansion cycle was  $10^{-3}$  torr or less. Six expansion cycles sufficed to reduce the concentration of oxygen in the suspension to a calculated value of  $2.7 \times 10^{-2} \mu\text{M}$ ; this level of oxygen concentration is known to be sufficiently low to give a radiation response characteristic of spores in anoxia. Spectrophotometric analyses carried out before and after the degassing routine of solutions of 2,3-butanedione at concentrations used in the irradiation experiments showed that loss of the agent as a consequence of these expansions was negligible. Vessels were closed under vacuum and maintained in this condition for anoxic irradiation, the contents being slowly stirred to keep the cells in uniform suspension during exposure to x-rays. For irradiation in the presence of oxygen, 3 ml. of the buffer suspension were held in contact with air in an irradiation vessel. A side-arm on the body of the vessel was open to the atmosphere, and the contents were stirred during irradiation. In addition to maintaining cells in suspension, stirring in this instance maintained equilibrium between the gaseous and liquid phases.

Dose-survival data covering at least four log-cycles of inactivation were obtained for each experimental condition. The data were fitted to the expression:

$$S = 1 - [1 - \exp(-kD)]^n,$$

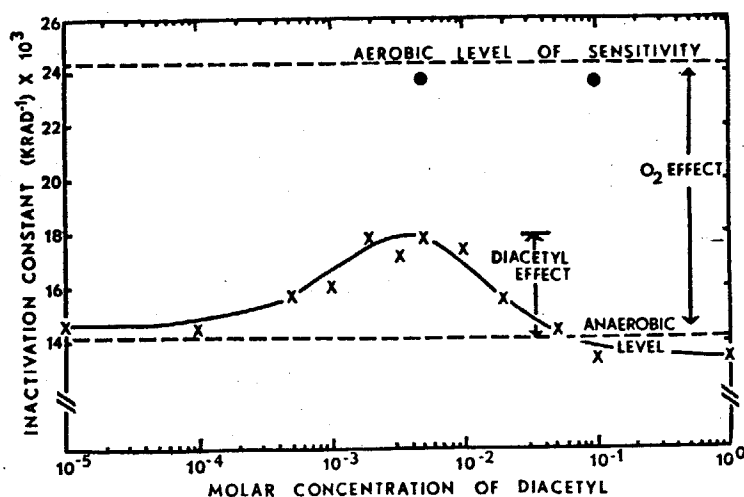
where  $S$  is the fraction of spores surviving a radiation dose  $D$ ,  $k$  is the inactivation rate constant and  $n$  is a constant giving a measure of the size of the shoulder of the dose-survival curve at low dose levels. Each survival curve was constructed from four points, with each point deriving from five plates. The best values for  $k$  and  $n$  for a given set of data were estimated by the iterative process described

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by Tyler and Dipert (1962). Only the value of  $k$  varied systematically with varying experimental conditions; its value is used here to describe numerically the effect of diacetyl on radiation sensitivity.

### Experimental results

The figure shows the effects of increasing concentrations of diacetyl on the radiation sensitivity of spores exposed anaerobically in aqueous suspension. As expected, at very low concentrations there is no increase above the anoxic level. At  $10^{-4}$  M, sensitivity begins to rise; at  $3 \times 10^{-3}$  M, it is at a maximum. At higher concentrations sensitivity falls, with anoxic sensitivity regained at about  $10^{-2}$  M. It is possible that the peak is a broad one extending from  $2 \times 10^{-3}$  to  $10^{-3}$  M.



The X's show the relationship between concentration of 2,3-butanedione (diacetyl) in aqueous buffer at pH 7.0 and sensitivity of spores of *Bacillus megaterium* to 50 kVp x-rays in oxygen-free suspensions. The solid points are sensitivities measured at the two indicated concentrations in suspensions saturated with oxygen.

The vertical bars are put on the figure to aid in visualizing the relative magnitudes of the effect of diacetyl in raising sensitivity above anoxic sensitivity and the corresponding effect of oxygen. The maximal effect of diacetyl in absolute units of radiation sensitivity is  $4 \times 10^{-3} \text{ krad}^{-1}$ ; while that of oxygen is  $10 \times 10^{-3} \text{ krad}^{-1}$ . The increase caused by diacetyl is but 40 per cent of the total oxygen effect. This recalls a proposal made earlier (Tallentire and Powers 1963) in which the total oxygen effect in the wet spore is composed of two kinds, one being 40 per cent the magnitude of the total oxygen effect (figure 6 (c) of Tallentire and Powers 1963).

The second general point shown in the figure is that the increase in radiation sensitivity brought about by diacetyl is within the oxygen effect. The solid points at  $5 \times 10^{-3}$  M diacetyl and at  $10^{-1}$  M were obtained from survival curves of spores irradiated in the presence of oxygen and of the compound at the indicated concentrations. These sensitivities are the equals of that obtained in oxygen alone; diacetyl is not seen to have any effect separate from or in addition to that

of oxygen. Previous questions are: is it necessary that diacetyl be present during irradiation, and is the effect associated with a state or species of short lifetime? These were tested in two kinds of experiments. (1) Spores were soaked prior to irradiation in  $5 \times 10^{-3}$  M diacetyl (the concentration giving maximal effect) for 30 min, then centrifuged three times in buffer, with 24 hours between each centrifugation. The radiation sensitivity in anoxia was that of spores not exposed to diacetyl; at any time, that is, no effect of the pre-treatment could be appreciated. (2) Spores and diacetyl solution ( $5 \times 10^{-3}$  M) were irradiated anoxically and separately; and then were mixed anoxically just at the end of the radiation interval. The survival of the spores was that expected of spores irradiated in buffer in the absence of oxygen. These experiments show that the effect of diacetyl is one that is realized only if the compound is present (within the time limit of our experimental procedure) with the spores at the time of irradiation.

Another question is that of possible toxicity of diacetyl. As a routine, spores were in contact with diacetyl at least 30 min before irradiation; extension of the contact time beyond this period (up to 1 week) had no further effect on radiation sensitivity. Control experiments run concurrently with irradiation exposures for the same times showed that the viability of unirradiated spores given diacetyl treatments was identical to that of untreated spores.

### 4. Discussion

The general results are that 2,3-butanedione increases radiation sensitivity of spores above the anaerobic level, that it is effective only when present during irradiation, and that it is ineffective as a sensitizer when irradiation is in the presence of oxygen. We interpret these results to mean that this compound operates within the oxygen effect in the spore system, and in doing so it acts on or with a short-lived state or chemical species. Our results allow the Adams and Dewey (1963) proposal that compounds of this type act by stabilizing radiation-produced electrons. We may also infer that this state or species is one of those acted upon by oxygen in increasing radiation sensitivity.

Of particular interest is the fact that the maximal increase in damage caused by diacetyl is only 40 per cent of the total oxygen effect in wet spores. One possible explanation is that oxygen is more efficient in its action than diacetyl is in copying it, or it might be that there are indeed two processes by which oxygen acts on spores irradiated in water suspension, only one of which can be accomplished by diacetyl. The involvement of oxygen in at least two radiation-induced processes was proposed by us several years ago (Tallentire and Powers 1963). The proposal was based on the actions of oxygen in spores of varying water content. One of these processes was thought to be responsible for 40 per cent of the total oxygen effect (the same fraction of the oxygen effect accomplished by diacetyl). At that time we allowed that the larger portion of the oxygen effect was due to the element of damage symbolized by  $k_{H_2O_2}$ ; for good experimental reasons (Powers 1966), we have called the  $k_{H_2O_2}$  element of damage the 'free radical' component for a number of years. In this  $k_{H_2O_2}$  element, damage probably induced by a peroxyradical formed from interaction of a radiation-produced free radical and oxygen. We might therefore expect that this element is independent of diacetyl presence. The 40 per cent effect we attributed to the  $k_{H_2O_2}$  element (originally defined as that damage seen in dry spores only when

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oxygen present during irradiation); in the paper describing our proposal, refrained from speculating about events responsible for  $k_{11}$ , but in view of the quantitative similarity pointed out in this paper, coupled with a consideration of the similarity in the electronic properties of oxygen and diacetyl, electronic affinity and stabilization could well be accounting for the sensitizing action of diacetyl in the wet spores, and could account also for one mechanism of action of oxygen.

Fortunately, this new proposal is open to test. As we reported earlier, the  $k_{11}$  element is strongly water-dependent (Tallentire and Powers 1963); it increases in magnitude as the water content of spores is decreased. Diacetyl exerts a considerable vapour pressure at ambient temperature, and hence we have the opportunity to test whether or not its effect shows a qualitative and quantitative similarity on this part of the oxygen effect with changes in the water content of spores. This series of tests is currently under way.

It is difficult to explain the drop in effectiveness of diacetyl to anoxic levels as concentration increases. At least two other studies on modifiers of radiation sensitivity have shown that effectiveness is related to concentration in this general manner. Dale, Davies and Russell (1961) showed that the radiation sensitivity of cells of *Shigella flexneri* at first increases to a maximum and then decreases (though not to the anoxic level) as the concentration of nitric oxide increases. With the spore of *Bacillus megaterium* in aqueous suspension, Russell (1966) showed that a steady increase in concentration of nitric oxide causes at first a rise, and then a decrease in radiation sensitivity; and in this instance at high concentrations of nitric oxide the sensitivity level is below that seen in anoxia. The explanations for these results are not recognized in terms of the radiation chemistry of aqueous solutions and cells; but these studies, together with the results presented here, point up the importance of concentration of solute on its action in modifying radiation sensitivity of biological organisms. The chemical explanations that suffice for dilute solutions are not necessarily those applicable in concentrated solutions, and full explanation of the radiation biology described above will be realized only after chemical study of irradiated concentrated solutions.

Le composé organique 2,3-butanédione (biacétyl) a une grande affinité pour les électrons libres et peut stabiliser les électrons produits par des radiations à haute énergie en solutions aqueuses. Dans la spore non-métabolique du *Bacillus megaterium* (Suspension-tampon à pH 7,0) ce composé augmente la sensibilité à la radiation en l'absence d'oxygène, lorsque la concentration augmente jusqu'à  $4 \times 10^{-3}$  M approximativement. L'augmentation maximum représente 40 pour cent de celle du total observé en présence d'un effet oxygène complet. Puisque le composé, en concentration maximum effective en présence d'oxygène, n'augmente pas la sensibilité à la radiation au-dessus du niveau observé dans l'oxygène seul, il est permis de penser que l'action du composé dans l'accroissement de la sensibilité représente l'une des actions de l'oxygène, c'est-à-dire, l'oxygène peut agir en partie comme un stabilisateur d'électrons. Au-dessus d'environ  $6 \times 10^{-3}$  M l'efficacité du composé en tant que sensibilisateur diminue, jusqu'à ce que le niveau anoxique de sensibilité soit atteint à  $6 \times 10^{-2}$  M. On ne propose, pour le moment, aucune explication concernant ce renversement de l'effet à haute concentration.

Die organische Verbindung 2,3-Butanedion (Diacetyl) hat eine starke Affinität für freie Elektronen und kann Elektronen stabilisieren, die von Hochenergiestrahlungen in wässrigen Lösungen erzeugt sind. In der nicht-metabolisierenden Spore von *Bacillus*

*megaterium* (Pufferlösung Suspension von pH 7,0), erhöht diese Verbindung die Strahlungsempfindlichkeit in der Abwesenheit von Sauerstoff bis zu einer Konzentration von ungefähr  $4 \times 10^{-3}$  M. Die maximale Erhöhung stellt 40 Prozent der bei vollem Sauerstoffeffekt beobachteten dar. Da die Verbindung bei maximal wirksamer Konzentration in der Gegenwart von Sauerstoff die Strahlungsempfindlichkeit nicht über das Niveau von Sauerstoff allein erhöht, ist es möglich, daß die potenzierende Wirkung der Verbindung eine der Wirkungen von Sauerstoff darstellt, d.h., Sauerstoff könnte teilweise Elektronen stabilisieren. Über ungefähr  $6 \times 10^{-3}$  M nimmt die Wirksamkeit der Verbindung als Sensibilisator ab, bis der Sauerstoff-freie Grad der Empfindlichkeit bei  $6 \times 10^{-2}$  M erreicht ist. Keine Vorschläge hinsichtlich dieser Umkehrung des Effektes bei hohen Konzentrationen werden gegenwärtig angeboten.

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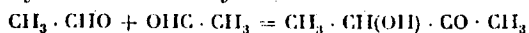
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Formation of the aroma-giving constituents in  
butter.

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It is generally held that the aroma of butter is chiefly due to the presence of diacetyl. Lactic acid bacteria, together with the aroma-producing organisms, form acetyl methyl carbinol and diacetyl apparently from acetaldehyde:



According to Hammer, citric acid is the constituent of milk from which acetyl methyl carbinol and diacetyl chiefly originate.

On analysing the starter or the ripened cream it is generally found that their content of diacetyl is very low or practically nil. On the other hand, acetyl methyl carbinol is present in considerable quantities when aroma-producing cultures are being used. If diacetyl is estimated in the usual manner, by distillation in the presence of ferric chloride, whereby acetyl methyl carbinol is quantitatively oxidised into diacetyl, it is natural that high values can be obtained for diacetyl although there might be none in the original sample. Since acetyl methyl carbinol is an odourless and tasteless compound, the above procedure is not suited for a quantitative estimation of butter aroma.

We have generally found no diacetyl on analysing samples of starters and ripened cream, although their content of acetyl methyl carbinol has been considerably high. Thus, for instance, 500 ml of the starter contained;

time, hours	diacetyl (as Ni-compound)	acetyl methyl carbinol (as Ni-comp. of diacetyl, mg.)
18	0	6.8
36	0	12.6
60	0	41.8
81	0	98.6
110	0	116.4

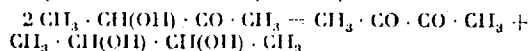
A distinct formation of diacetyl took place first after air or oxygen was passed through the culture during the ripening process. This is shown by the following table.

Treatment	time, hours	diacetyl (as Ni-comp. pound, mg)	acetyl methyl carbinol (as Ni-comp. of diacetyl, mg.)
O <sub>2</sub> passed through the ripening culture three times	18	19.0	not determ.
O <sub>2</sub> passed through the culture five times	42	22.1	10.1

No diacetyl was found when oxygen was led into the starter immediately before the determination of

diacetyl. The above results are therefore not attributable simply to a direct oxidation of acetyl methyl carbinol. When the cultures were aerated the results were similar to those obtained with oxygen. Treatment with CO<sub>2</sub> or N<sub>2</sub> prevented the formation of diacetyl.

We may thus conclude that diacetyl is not formed through an oxydo-reduction process between two molecules of acetyl methyl carbinol with a simultaneous production of butylen glycol:



but only when oxygen is acting as a hydrogen acceptor:  
 $2 \text{CH}_3 \cdot \text{CH(OH)} \cdot \text{CO} \cdot \text{CH}_3 + \text{O}_2 = 2 \text{CH}_3 \cdot \text{CO} \cdot \text{CO} \cdot \text{CH}_3 + \text{H}_2\text{O}_2$

The fact that diacetyl is thus formed only in the presence of oxygen should be duly considered in the preparation of butter. The cream should therefore be ripened under as aerobic conditions as possible and the churning carried out so as to ensure a maximum content of air in the butter.

There exist numerous patented methods according to which the churning of the cream is done in a churn filled with some indifferent gas. Such methods are obviously not suited for the production of butter with a rich aroma.

In our experimental creamery we have made several attempts to increase and improve the aroma of butter by passing sterile air into the cream during ripening. This method was found to have a very favourable effect on the aroma of the fresh butter, the diacetyl content of which was thereby considerably increased.

During storage, however, the differences between the aromas of the aerated and the control butter generally lessened, although the deterioration of the aerated butter was not more extensive than that of the control. After a month's storage both samples were graded approximately equal.

Similar results were obtained from experiments in a big creamery. The aroma of the fresh butter can thus be considerably improved by aeration of the cream. Such butter should, however, be sold fresh (within a week or so), in order to make full use of its rich aroma.

It may also be mentioned here that, if the deterioration of butter is due to micro-organisms (e. g. bacteria of the *fluorescens* group), the butter loses its aroma and diacetyl content very quickly, before any other defects become detectable.

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# THE EFFECT OF IODOPHORS ON THE FLAVOUR OF MILK AND ON THE ACTIVITY OF LACTIC CULTURES

Ireland, Dep. Agr. Fish & J. 64:128-33, (1967)

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Iodophor detergent sterilizers are now widely used for cleaning and sterilizing milking equipment and utensils. Jensen et al (1) found that off flavour in milk could be induced by iodophor-soaked milking machine inflations and reported lower flavour threshold values for iodophors in milk (4.5 ppm.) than those previously reported in the literature. The lowest concentrations of iodine that could be detected organoleptically by Gaida et al (2) were 6 to 8 ppm. in skim milk and 10 to 14 ppm. in whole milk. Johns (3) found that concentrations of iodine up to 10 ppm. in milk failed to impart an off flavour either before or after pasteurisation. Later Johns and Berzins (4) found that when 8 ppm. of iodine from one iodophor and 16 ppm. from another iodophor was added to milk, an off flavour developed. Swartling (5) considered that amounts of iodine from iodophors likely to get into milk could not be expected to have any adverse effects.

Another problem associated with the presence of residual dairy sterilizers in milk is that of inhibition of lactic cultures for cheese-making. Although the levels of chlorine in milk having an inhibitory effect on starters have been determined (6) (7), literature on the levels of iodine which may inhibit or prevent acid production by starters is not available.

This study was undertaken to determine the levels of available iodine from iodophors which would affect the flavour of milk and the activity of a commercial lactic culture.

## METHODS AND MATERIALS

Three commercial iodophors A, B, and C which were compounded in the Irish Republic, were used in this study. The percentage available iodine of each iodophor was determined by titrating 20g. of iodophor concentrate, diluted to 50 ml. with distilled water with 0.1 N sodium thiosulphate solution to a colourless end point. The percentage of phosphoric acid in each iodophor was determined by potentiometric titration using 0.1 N NaOH and checking for the presence of other acids by continuing the titration to the second equivalence point.

*Flavour threshold levels of iodophors in milk.*—Solutions of each iodophor were prepared to give concentrations of available iodine from 0 to 20 ppm. in milk samples. The levels at which the different iodophors were detectable in milk were determined by a taste panel of 10 judges.

*Activity of a lactic culture.*—Sterilized tubes of skim milk were inoculated with iodophors, A, B and C to give concentrations of available iodine ranging from 0 to 100 ppm. Ten replicate samples at each concentration level were prepared. Each tube was inoculated with 2% of commercial lactic culture and incubated at 73°F. A sample at each different iodine concentration level was tested for pH before incubation, and fresh samples were taken from the incubator for testing at 2 hourly intervals thereafter. The pH was measured electrometrically. Similar procedures were used when studying the effect of iodophors B and C on lactic cultures.

*Acetyl-methylcarbinol and diacetyl production.*—After determining the pH, the acetyl-methylcarbinol plus diacetyl content of each sample was determined using a modification of the method outlined by King (8). When using the method of King, turbid filtrates were sometimes obtained. In this study one ml. of each filtrate was diluted threefold with M/50 diamino-ethane-tetra acetic acid solution. This procedure gave clear solutions and enabled the intensity of the lilac-red colour of the filtrates produced by acetyl-methylcarbinol and diacetyl to be measured with a Spectronic 20 photoelectric colorimeter. A wavelength of 250 mμ. was used. A standard curve for diacetyl in milk was established using these procedures with samples of fresh milk to which various known quantities of diacetyl were added. The acetyl-methylcarbinol plus diacetyl contents of the test samples were expressed as mg. of diacetyl per 100 ml. of milk.

## RESULTS

Results of the analyses of the iodophors are given in Table 1.

Table 1

Phosphoric acid and available iodine contents of the iodophors.

Iodophor	% Phosphoric acid	% available iodine w/w	% available iodine w/v
A	31.3	1.90	2.20
B	13.1	2.15	2.29
C	17.0	1.89	2.03

The phosphoric acid content of the three iodophors varied widely. Differences in the percentages of available iodine content of the iodophors were not large.

*Flavour threshold levels.*—The number of judges who could detect the iodophors in milk at different iodine concentration levels is given in Table 2.

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Table 2

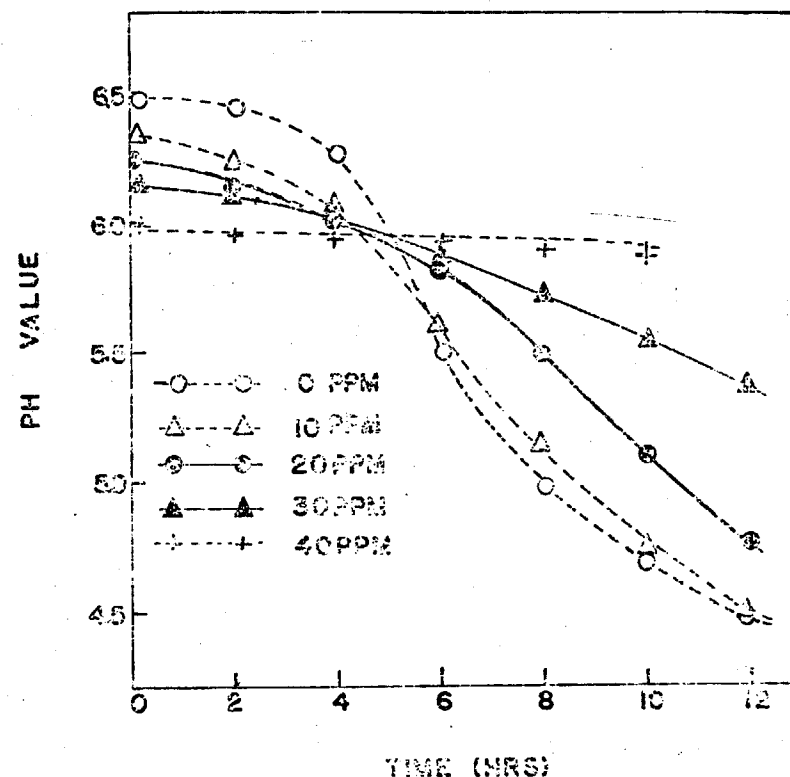
Number of judges who could detect an off flavour in milk samples at different iodine concentration levels.

Iodophor	Iodine concentration (ppm.)						
	2	4	6	8	10	12	14
A	0	0	0	3	6	9	10
B	0	1	6	8	10	10	10
C	0	0	2	5	7	10	10

The flavour threshold values varied for the different iodophors. The concentration level of iodine at which different judges could detect an off flavour in milk also varied. In general, a concentration of iodophor in milk sufficient to give 6 to 10 ppm. available iodine might be expected to give an off flavour to the milk, but concentrations of iodine up to 4-5 ppm. may well escape organoleptic detection.

**Inhibition of lactic cultures.**—The results of an experiment to determine the level of available iodine from iodophor A which would inhibit acid production by a commercial lactic culture (Hansen's Laboratory, Denmark) are given in Figure 1.

FIGURE 1



Changes in pH of milk samples containing 2% lactic culture and different levels of iodine, with time of incubation at 73°F.

Addition of iodophor to milk samples lowers the pH values. At a concentration of 10 ppm. available iodine the activity of the lactic culture at 73°F. was slightly retarded. After twelve hours at this temperature, however, the control sample and the sample containing 10 ppm. iodine had the same pH value. At iodine concentration levels greater than 10 ppm. inhibition of the lactic culture was increased until at 40 ppm. no change in pH was observed after 10 hours. At 5 ppm. available iodine detectable inhibition of the culture by this method did not occur.

The addition of iodophors B and C to milk samples did not lower the pH of the samples to the same extent as did corresponding amounts of iodophor A. The general patterns of the pH time curves for lactic cultures containing iodophors B and C at levels of available iodine from 0 to 100 ppm. were similar to curves shown for iodophor A.

**Acetylmethylcarbinol and diacetyl production.**—The inhibitory effect of different levels of iodine in milk on the production of acetylmethylcarbinol and diacetyl by a lactic culture is shown in Table 3.

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Table 3

Acetylmethylcarbinol and diacetyl production (expressed as mg. diacetyl/100 mls. of milk) by a lactic culture at 73°F. in the presence of different levels of iodine.

Conc. of iodine ppm.	Time Hr.				
	2	4	6	8	10
0	0	.26	1.66	5.83	5.85
5	0	.24	1.00	6.83	6.83
10	0	.24	.75	4.75	6.83
20	0	0	.25	3.91	6.83
30	0	0	0	1.83	2.83
40	0	0	0	.58	.83
50	0	0	0	.08	.66
60	0	0	0	0	.33
80	0	0	0	0	0
100	0	0	0	0	0

Acetylmethylcarbinol plus diacetyl production, expressed here as mgs. of diacetyl per 100 mls. of milk, was delayed by the presence of 20 ppm. of available iodine in milk and 60-80 ppm. of iodine caused complete inhibition of acetylmethylcarbinol plus diacetyl for 10 hours.

Problems of off flavour in milk and inhibition of commercial lactic cultures for cheese-making due to the presence of residual iodophors in milk when iodophors are used as detergent sterilizers for dairy equipment and utensils, are not likely to arise if iodophors are used with reasonable care. If we consider the recommended use concentration of the iodophors studied, which corresponds to an iodine concentration of about 30 ppm. of available iodine and the recommended after rinse concentration of about 12 ppm. available iodine, the possibility of residual iodophor from equipment causing off flavours in milk and inhibition of lactic starters for cheese-making is remote.

## ACKNOWLEDGMENT

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(2263)

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## CORRELATION BETWEEN GAS-CHROMATOGRAPHIC PATTERNS AND FLAVOR EVALUATION OF CHEMICAL MIXTURES AND OF COLA BEVERAGES

**SUMMARY**—Two methods of discriminant analysis, Chi-square tests, "t" tests and analysis of variance were applied to gas-liquid chromatographic (GLC) data to compare GLC measurements of volatiles with organoleptic evaluation of flavor. Model systems composed of ethyl butyrate, heptylate and benzaldehyde were used for one set of trials. A second system consisted of blends of Pepsi-Cola® and Coca-Cola®. The stepwise discriminant analysis (SDA) procedure described by Powers et al. (1968) was only moderately successful in classifying the mixtures when applied to the GLC data for the model systems, but the cola blends could be classified readily. From GLC data, cola blends differing from each other by only 7–10% could be distinguished. A new discriminant analysis program, designated MUDAID by its originator (Bargmann, 1967), is more useful for predictive purposes. The analysis yields the correlation coefficients for each peak with sample differences and the weighting factors. Discriminant equations can thus be written. Substitution of measurement values for unknowns, their identity may be predicted. Organoleptic subthreshold additive effects could not be demonstrated with binary mixtures of flavor substances, but judge-compound interaction effects were observed.

### INTRODUCTION

THE IDEA of using gas-liquid chromatography (GLC) for evaluation of the flavor of foods is nearly as old as GLC itself. Powers (1968) pointed out that flavor evaluation by GLC has not attained the status it might have because suitable methods of correlating complex GLC data with sensory evaluation have been lacking. Powers et al. (1968) showed that stepwise discriminant analysis (SDA) could be used to classify foods for flavor from GLC data.

The present study was undertaken to determine 1) whether assessment of differences by the SDA method could be done at closer intervals than by organoleptic evaluation; 2) whether alternate methods of using GLC data efficiently for flavor evaluation could be found; and 3) whether subthreshold additive effects, as mentioned by Keith et al. (1968), result from sensitivity of particular judges to specific compounds or are truly additive. It has a bearing on correlation, since GLC is a separation process, whereas organoleptic assessment of flavor is an integrative process.

### REVIEW OF LITERATURE

OSTROM (1967), Baker et al. (1967), Powers et al. (1968) and Powers (1968) reviewed much of the literature regarding comparisons between GLC measurements and sensory evaluation of flavor.

In foods, simple correlation studies are suitable because one or a few components are closely correlated with flavor quality (Powers, 1968). Hammond et al. (1964), Arnold et al. (1966) and Kings et al. (1968) have each described flavor defect in milk or cheese resulting from a single compound. Powers et al.

(1968) cited other examples of simple correlation effects.

More often, the true flavor or off-flavor of a food must be attributed to several compounds. Aurand et al. (1965) found that the flavor of pickles is due to a blend of volatile compounds rather than the presence or absence of a single component. Buttery et al. (1967) reported that American-grown hops of different varieties could be distinguished by the relative percentages of certain components. MacLeod et al. (1966) stated that chromatograms of lemon oils from throughout the world were very similar and that differences were primarily quantitative rather than qualitative. Wong (1963) observed that GLC differences between fresh and decomposed cream were primarily quantitative. Sevenants et al. (1966) concluded that typical peach aroma was probably an integrated response to a wide spectrum of compounds and, in fact, none of the individual compounds was at all peachlike in aroma.

Miller (1966), Stephens (1966), Keith (1967), Buttery et al. (1967), de Becze et al. (1967), Hawkes et al. (1967) and Powers et al. (1968) have each attempted to compare, upon some mathematical or statistical basis, flavor differences with chromatographic differences. Henis et al. (1966), O'Brien (1967), Dravnieks et al. (1967) and MacGee (1968) have carried on similar studies to identify from GLC data different species or strains of microorganisms or of other biological entities.

### EXPERIMENTAL

#### GLC vs. organoleptic comparisons

Two types of trials were used to compare GLC and organoleptic evaluation. One consisted of a model system and the other of blends of Pepsi-Cola® and Coca-Cola®. Ethyl butyrate, ethyl heptylate and benzal-

dehyde were dissolved in double-distilled water at concentrations of 2.272, 1.664 and 5.125 mg/liter to form a control solution. Two of the compounds were then held at these levels and the third compound reduced in concentration until the resulting mixture could be distinguished from the control solution by a six-member taste panel. The same triangular method of sensory evaluation was used by Keith et al. (1968).

For the GLC analysis, 500-ml portions of the solutions were extracted in a distillation-extraction apparatus (Likens et al., 1964). Approximately 40 ml of pentane was used as the solvent, and the solutions were extracted for 2 hr. The solvent flask was then stored at 10°F for not less than 2 hr to freeze out dissolved or emulsified water. The pentane was decanted and then concentrated in a Kuderna-Danish evaporator partially submerged in a water bath at 37°C. The pentane extract was reduced to 60–70  $\mu$ liters. The concentrated sample was held in an ice bath while GLC runs were being made; 5- $\mu$ liter portions were used for chromatographic injection.

The chromatographic packing consisted of 10% Triton X-305 (alkyl aryl polyether alcohol) coated on to 60-80-mesh Diatoport S. The columns were 20 ft, 1/4-in. stainless steel, and a dual-column gas chromatograph (F & M Model 810) was used. The programming sequence consisted of a 5-min post-injection interval at 160°C, followed by programming at 6°/minute to 210°C. The injection port temperature was 295°C; the detector temperature was 275°C, and the nitrogen, hydrogen and oxygen flow rates 30, 63 and 150 ml/minute, respectively.

The areas of the various peaks were measured with a planimeter and each area converted to the percentage it represented of the total for the areas of all peaks (Powers et al., 1968).

#### Cola-beverage trials

Blends of Pepsi-Cola and Coca-Cola were prepared in ratios of 67:33; 60:40; 50:50; 40:60 and 33:67. Using the triangular procedure, the panel was then asked to select the odd sample in comparisons involving Pepsi-Cola, Coca-Cola or different blend ratios. The panel was also asked to place 4 solutions in order. They were 100, 67, 50 and 33% of each cola beverage.

The various blends were extracted as described for the model system, except 450 ml of the cola solutions were extracted, ethyl ether was used as the solvent instead of pentane and the extraction period was 1 instead of 2 hr. One further difference was that 0.1  $\mu$ liter of  $\gamma$ -undecalactone was injected into the concentrated extract just before gas-chromatographic analysis, to provide a reference compound for retention times and area. For statistical purposes, the measured area of each peak, the area transformed to percentage and the ratio of the area to the area of the reference compound were thus available.

Table 1—Minimum change<sup>1</sup> in 1 compound necessary to enable solutions to be distinguished organoleptically.

Compound	Solution A (Control)	Solution detectably different		
		B	C	D
Ethyl butyrate	2.272	1.717	2.272	2.272
Ethyl heptylate	1.664	1.664	0.422	1.664
Benzaldehyde	5.125	5.125	5.125	1.294

<sup>1</sup>Concentrations are in milligrams/liter.

Table 2—Differentiation<sup>1</sup> of cola beverages by organoleptic evaluation for flavor.

Cola beverages and blends						
100% Pepsi-Cola	67P:33C	60P:40C	50P:50C	40P:60C	33P:67C	100% Coca-Cola

<sup>1</sup>Any 2 treatments not underlined by the same line are significantly different.

### Statistical methods

The same stepwise discriminant analysis procedure (Sampson, 1967) as used by Powers et al. (1968) was applied to the data. They observed some evidence of subthreshold additive effects, but after the panel was already disbanded they wondered whether the subthreshold additive effects observed resulted from the sensitivity of a particular judge for a particular compound, rather than 2 or more compounds, below their levels of detectability, augmenting each other so as to make their presence detectable.

For these trials, a 12-member panel was used and the compounds were ethyl acetate, benzaldehyde, 2-methyl butyric acid, isovaleric aldehyde, amyl butyrate and  $\gamma$ -undecalactone. The threshold levels reported by Keith et al. (1968) were used as a starting point. By increasing or decreasing the concentration, the threshold level for the panel was found. This, in effect, was a pooled Chi-square test. In conformity with Keith et al. (1968), the 0.05 level of significance was used. Each panelist was also tested individually to establish his threshold level for each compound. Binary mixtures of these 6 compounds were then prepared for sensory evaluation with each compound at 50% of the threshold level established by the panel. The theoretical threshold was, thus, 100% of the panel threshold.

To learn whether significant results came from judge-compound interactions or were truly additive, the panel was split into 3 groups. Judges whose threshold was neither greater nor less than that of the panel were put into Group A. The most sensitive judges were put into Group B.

Judges in Group A were given solutions with each compound at 50% of their (and the panel's) threshold levels. Judges in Group B were given solutions with the two compounds at 75 or 67% of the judge's respective threshold levels. For each judge, the sum naturally exceeded 100% of his theoretical level, but the sum was set to approximately 100% of the panel's threshold level.

Since there were 6 compounds and a judge might be sensitive for 1 compound but not for another, a judge sometimes was in Group A, Group B or temporarily eliminated according to the particular binary combination under study and his sensitivity to these compounds.

## RESULTS & DISCUSSION

### Organoleptic evaluation

The model system was used in the hope that a simple system would facilitate the development of means of correlating sensory and objective evaluation. This turned out not to be so. Rather sizeable differences had to exist in the concentrations of the 3 chemicals before the change became organoleptically detectable. Recorded (Table 1) are the minimal changes necessary for the panel to detect a significant difference as one compound at a time was decreased in concentration. For ethyl butyrate, ethyl heptylate and benzaldehyde the respective decreases had to be 24.5, 74.6 and 74.7% before the panel could tell the difference against the background of the 2 other chemicals. Though only 3 solutions were tested at a time, the harsh chemical flavor of some combinations over-rode organoleptic differences.

The panel proved itself to be moderately sensitive in detecting differences among the cola solutions. It could detect differences when the blends varied by 17% but not when they varied by 10% or less. Results are listed in Table 2.

### SDA analysis

Of 195 chromatograms available from the same solutions as listed on Table 1, 13% of the solutions were erroneously classified by the SDA procedure from the GLC data. The SDA procedure applied to the data for the cola solutions was remarkably effective. Compositional differences to within 7% could be resolved (see Table 3).

Table 3 is a classification based upon using the percentage peak areas of peaks 4, 5 and 6 as denominators. The procedure followed was the same as that of Powers et al. (1968). First, the peak areas were transformed to percentage peak areas. Then all possible simple ratios were formed using each percentage peak area

as the denominator. The SDA program was written to handle only 80 variables at a time. As there were 24 peaks for the cola solutions, the data were subdivided into groups consisting of 3 peaks as denominators. The number of steps required to yield correct classification by each of the 8 groups is shown in Table 4. Shown also is the number of steps required when the 9 most effective ratios from each of the 8 subgroups were combined.

That a composite of the 72 most effective ratios from each of the 8 subgroups required more steps than most of the subgroups needs explanation. Stepwise discriminant analysis depends upon the intercorrelations between variables and the degree of dispersion of each variable. In generating 72 ratios with 9 different denominators instead of 3, heterogeneity apparently was increased.

One question arises: Rather than form ratios, can discrimination be effected from the peak areas themselves? This could not quite be done for the cola solutions. 69 out of 70 chromatograms were correctly classified as to blend, but 1 chromatogram for a 33:67 blend was incorrectly classified as a 40:60 blend. Powers et al. (1968) did not try to classify their coffee chromatograms from percentage peak heights. Their data were subjected to the SDA procedure except the 33 percentage peak heights were used instead of ratios. 1 peak area alone was sufficient to classify the coffee. This demonstrates that for some applications there is no need of forming ratios from the peak areas before the SDA procedure is applied. In trials to be reported, Milutinovic et al. (1970) were able to discriminate among samples without resort to ratios. In this study, formation of ratios was necessary for the model system and the cola beverages, if one sets a standard of 100% success. Actually, successful classification of 69 out of 70 chromatograms without resort to the formation of ratios is entirely acceptable. Upon a probability basis, one should not expect every chromatogram out of 70 to be always classified correctly.

Concerning ratios, a recent patent (Mahlmann et al., 1969) is pertinent. The patent points out that the process leads to flavorful soluble coffee and the flavor is defined as having a unique GLC spectrum. The spectrum is given in terms of ratios among volatiles.

### Noncomputer methods

1 limitation of the SDA program is that a computer is required. Thought was given to the possibility of utilizing all the data from relatively complex chromatograms without having to resort to high-speed computing. 3 different methods of hand calculation were tried: 1) analysis of variance combined with Duncan's multiple range test; 2) a pooled Chi-square test

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and "3) a group "t" test. For the last-named procedure, the significance of the number of significant "t" tests was estimated from an upper and lower binomial-confidence-limits table (Mainland et al., 1956).

Analysis of variance and Duncan's multiple range tests were moderately effective for the model solutions. One has to make a decision from the multiple range test for each peak area separately. There is risk of error in each decision and these risks are cumulative. Actually, for only 3 peaks this was no real problem. Table 5 shows Duncan's multiple range tests for 8 of the 24 peak areas of the cola trials. One can see that if one had measurement values for an unknown, one could probably decide to which group the sample belonged.

Chi-square analysis also could be used. This is unorthodox, for the Chi-square test is normally used for "expected" values, generally discontinuous, such as expected number of progenies in genetical experiments. By Chi-square tests of one group against another, just about the same peaks were picked as being critical to discrimination as by the analysis of variance. When group "t" tests were used for the same purpose, there was good agreement, too, as to the peaks most useful in distinguishing among the 7 cola treatments. Young (1968) correctly identified 5 out of 7 unknown blends by using the Chi-square procedure.

The analysis of variance indicated there was significant interaction between peak areas and treatment. Some peaks were useful to separate groups at one end of the blend series, but not at the other end (see Table 5). Curvilinearity of most of the regression lines accounted for much of the interaction.

#### Multiple discrimination

The techniques of regression and stepwise discrimination are primarily concerned with the problem of classifying individual chromatograms in terms of

Table 3—Classification of cola beverages from ratios of percentage peak areas involving peaks 4, 5 and 6 as denominators.

Solution	Classification					
	100%	67P:33C	60P:40C	50P:50C	40P:60C	33P:67C
100% Pepsi-Cola	9					
67P:33C		10				
60P:40C			10			
50P:50C				9		
40P:60C					12	
33P:67C						11
100% Coca-Cola						9

their similarities to the groups of chromatograms for each treatment in the present study. Since a new discriminant function is established for each pair of observed compounds, it would not be easy to generalize such results to a class of, say, all cola beverages. For this latter problem, a multiple discriminant analysis on peak areas as response variables seems more appropriate. The MUDAID program (Bargmann, 1967) performs such an analysis in one of its phases. Each variable, i.e. peak area, is studied by an irregular 2-way classification-design analysis. Adjusted mean values for replications and treatments are calculated as well as F values for replication, treatment and interaction effects. This univariate pass for each peak area is used primarily to detect experimental outliers or recording errors, which invariably result in significant interaction effects.

A second run is then made on the edited data. The F values for treatments for each peak are valuable indicators of the degree of discrimination due to each peak. A multiple discriminant function is then calculated (largest eigen vector of a matrix product) which is, in fact, a linear combination of the area under each peak.

It is an artificial variable or score given to each compound, with the weights chosen in such a way that the distance between treatments is maximized. The program then proceeds to calculate correlations between each variable (peak area) and the best discriminant function. This is thus a measure of proximity of each peak area to the best discriminator. If some peak areas show low correlation, they obviously do not contribute to the over-all discrimination between treatments.

The variables with low correlation are eliminated and a second or third run is made on the remaining peak areas. Finally, only those peaks having the highest F values for treatment effects and the highest correlations with the over-all best discriminator are retained.

The discriminant function for 9 of the 24 variables of the cola data was:

$$Z = 0.673 \times_4 + 14.7 \times_7 - 9.68 \times_8 + 2.56 \times_{10} + 0.748 \times_{11} + 9.4 \times_{12} - 1.02 \times_{13} + 24.6 \times_{15} - 10.6 \times_{21}$$

The respective correlation values, in the order of peaks just listed were -0.194, 0.257, 0.136, 0.289, 0.176, 0.294, -0.187, 0.104 and -0.156. Peak 12 was

Table 4—Number of steps required to classify chromatograms of cola solutions.

Peaks used as denominators	No. steps to successful classification
1, 2, 3	24
4, 5, 6	19
7, 8, 9	24
10, 11, 12	39
13, 14, 15	19
16, 17, 18	24
19, 20, 21	29
22, 23, 24	34
Composite <sup>1</sup>	39

<sup>1</sup>The composite consisted of the 9 most efficient ratios from each of the 8 trials.

Table 5—Peaks selected by analysis of variance and Duncan's multiple range test as being useful to distinguish among cola solutions.<sup>1</sup>

Peak	100P	40:60	50:50	67:33	33:67	60:40	100C
2	100P						
4	100P	67:33	40:60	60:40	50:50	33:67	100P
7	100C	40:60	33:67	50:50	60:40	67:33	100P
10	100C	33:67	60:40	50:50	40:60	67:33	100P
11	100C	33:67	40:60	60:40	50:50	67:33	100P
12	40:60	50:50	100C	60:40	33:67	67:33	100P
13	100P	67:33	60:40	50:50	40:60	33:67	100C
21	100P	67:33	60:40	50:50	40:60	33:67	100C

<sup>1</sup>The first figure in each ratio designates the percentage of Pepsi-Cola.

most highly correlated with treatment change. The degree of dispersion enters into the weighing values as well as correlation. Peaks with the highest correlation to the best discriminating equation are likely to be involved in flavor. If they are not flavor substances themselves, they affect flavor in such subtle ways as by sub-threshold additive effects, reaction with other components or through dilution of those compounds which are odorous.

Table 6 lists the 9 variables chosen and the Z values calculated for each cola treatment. To predict unknowns, one merely substitutes in measurement values for each of the variables and calculates the Z value. From this value one can estimate to which group the unknown belongs.

Comparison of Tables 5 and 6 shows that nearly the same peaks were selected as being important to discrimination by the analysis of variance procedure as by the discriminant analysis method. As already pointed out, the Chi-square and group "t" tests were in agreement with the analysis of variance procedure. Both tables also show the virtual impossibility of discriminating between the 50:50 and 33:67 solutions on the basis of chromatograms. This illustrates the overly optimistic results obtained by internal

Table 6—Values<sup>1</sup> of components of the discriminant function for cola mixtures.

Peak No.	100P	67:33	60:40	50:50	40:60	33:67	100C
4	-0.064	-0.055	-0.053	-0.052	-0.055	-0.046	-0.037
7	0.021	-0.044	0.060	0.076	0.100	0.081	0.113
8	-0.028	-0.015	-0.016	-0.013	-0.010	-0.014	-0.001
10	0.204	0.244	0.258	0.256	0.254	0.266	0.304
11	0.142	0.160	0.174	0.170	0.177	0.178	0.198
12	0.001	0.001	-0.047	0.066	0.085	0.042	0.062
13	-0.055	-0.048	0.047	-0.041	-0.039	-0.035	-0.023
15	0.020	0.027	0.022	0.022	0.020	0.014	0.002
21	-0.034	-0.030	-0.029	-0.020	-0.014	-0.013	-0.010
Z Value	0.207	0.328	0.415	0.464	0.518	0.473	0.608

<sup>1</sup> Each value is the product of peak area times weighing factor.

<sup>2</sup> The first figure in each blend ratio designates the percentage of Pepsi-Cola.

validity checks. Note that, in the pairwise discrimination, these 3 solutions were almost always correctly classified. The single multiple discriminant function which permits application to unknown substances is more indicative of the quality of discrimination between future chromatograms.

#### Limitations and problems

Failure of the model system to be as amenable to SDA analysis as the cola solutions needs to be explained. 3 problems were observed: Use of the percentage area transformation was inappropriate for the model system since it contained only 3 variables. Whenever one compound was reduced in concentration—and thus in peak area—the areas of the 2 other compounds were automatically increased upon the percentage basis. With our method of analysis, this produced an error component. A symmetrical complementation design (Beyer, 1961) would have permitted a more sophisticated analysis to be performed.

When the transformation was not made, then error as a result of extraction, column and instrument variation was more obvious. This represented a second type of error. Analysis of variance demonstrated that when only 3 compounds were present, the percentage efficiency of extraction changed as concentration changed. No attempt was made to check

extraction efficiency of the cola beverages because the original concentrations naturally were not obtainable. From observation of the variance for each of the 24 peaks, distillation efficiency of each component of the 24-component system was more stable than in the 3-component system. Even from the extremes of Pepsi-Cola and Coca-Cola the differences in volatile components were less drastic than for the model systems. Figure 1 shows chromatograms for Pepsi-Cola, Coca-Cola and a 50:50 blend of each.

A third source of error was differential volatilization from the extraction concentrate. If 5 or 6 injections were made from the same extract, the first peaks gradually decreased in size, suggesting that the more volatile substances were escaping from the solution. The concentrate was kept in small sealed vials, at 10°F when not in use, in ice water between injections. As cumulative sample withdrawal became appreciable, apparently new liquid:headspace equilibria were established. Again, in the 3-component system this was more serious than in the 24-component extract.

#### Organoleptic subthreshold additive effects

Threshold values for the panel and the most sensitive members of the panel are shown in Table 7. Among the 30 possible combinations of the 6 compounds listed

Table 7—Threshold values for compounds used in subthreshold additive effect study.

Compound	Threshold concentration <sup>1</sup>	Threshold concn of most sensitive panel member
2-Methyl butyric acid	1.27	1.02
Ethyl acetoacetate	5.20	2.08
Benzaldehyde	2.35	1.41
Isovaleric aldehyde	0.28	0.17
Amyl butyrate	1.29	0.77
Gamma-Undecalactone	0.15	0.12

<sup>1</sup> Concentrations are in parts per million.

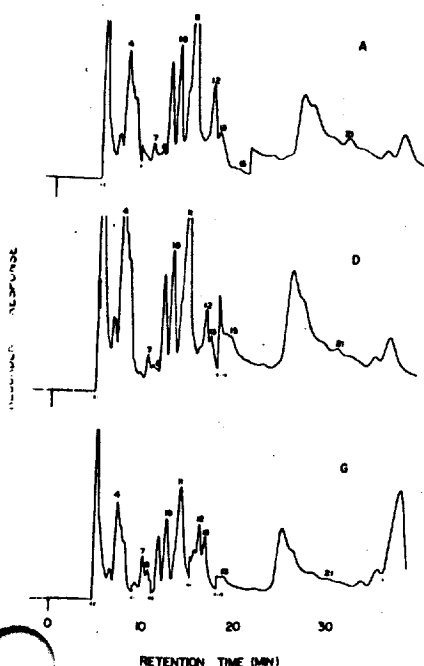


Fig. 1—Chromatograms of cola solutions. A = Pepsi-Cola, B = Coca-Cola; C = a 50:50 blend of each cola beverage. The last peak on chromatogram C is the reference compound,  $\gamma$ -undecalactone. The peaks, which are numbered, are the same as those used in the discriminant equation. Before calculation, areas were adjusted to compensate for attenuation changes.

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Table 7, only 5 combinations could be used for Group A with the limitation that each judge's sensitivity equal the panel average. 6 combinations were suitable for the Group B trials. None of the 5 combinations for the Group A trials showed a statistically significant subthreshold additive effect. Judge-compound interactions were detected. 1 panel member, for example, could consistently pick out the amyl butyrate + ethyl acetoacetate mixture, and another member could detect the benzaldehyde + amyl butyrate mixture. The same thing was true for the Group B trials. No combination showed a statistically significant subthreshold additive effect when judge-compound interaction was removed.

The trials were not intended to disprove that subthreshold additive effects exist. Logically, they must exist. The trials affirmed the reservation of Keith et al. (1968) that precautions need to be taken to separate judge-compound interaction in attempting to demonstrate subthreshold additive effects. Whether flavor differences are detected through a subthreshold additive effect or the sensitivity of a particular individual for a particular compound, there are other factors which complicate organoleptic-objective correlations more seriously.

To cite only a few, no extraction procedure removes the volatiles 100%. At whatever levels of efficiency they are expected, the ratio among volatiles is upset. At the GLC stage, the chromatographic pattern is determined by the column and the operating conditions. These and many other factors mean that correlation is restricted to particular conditions of sample treatment and analysis. As pointed out by de Bezze et al. (1967) no single column or operating condition is satisfactory for all components. Upon adding to 100° proof ethanol pure components known to be in Bourbon, de Bezze et al. reported that there was no resemblance in either odor or taste between the synthetic mixture and Bourbon. These investigators used 4 columns to separate several types of alcoholic beverages. Notwithstanding lack of compatibility between human sensors and GLC detectors, objective analysis of GLC data has much to com-

mend it. Though our panel could generally select the odd cola solution from the 2 identical samples, it invariably failed in attempts to line up correctly 67:33, 50:50 and 33:67 blends, whether Pepsi-Cola or Coca-Cola was the anchor beverage at one end of the series. This could be done readily by either of the discriminant analysis procedures tried.

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